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Functional characterization of Cl^-/HCO_3^- exchange in villous cells of the mouse ileum

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ABSTRACT

At least three kinds of Cl^-/HCO_3^- exchangers, SLC26A3, SLC26A6 and AE2, have been demonstrated to be expressed in the intestinal epithelial cell. To examine the functional expression of these exchangers in the native enterocyte, we studied the Cl^-/HCO_3^-- exchange activity in isolated villi from the mouse ileum by microfluorometric intracellular pH (pH_i) measurement. The pH_i value increased upon Cl^- removal when the villus was superfused with an HCO_3^-/CO_2-buffered solution, while the response was blunted when superfused with an HCO_3^-/CO_2-free, Hepes-buffered solution. The recovery of pH_i value induced by Cl^- re-addition (after initial Cl^- removal) was totally or partially mimicked by the addition of Br^-, I^-, F^-, NO_3^-, or SO_4^{2-} (in the absence of Cl^-). The increase in pH_i value induced by Cl^- removal was partially inhibited in the presence of DIDS (30 μM), tenidap (10 μM), niflumic acid (30 μM) or NPPB (30 μM). Increasing the K^+ concentration from 5 mM to 60 mM in the superfusion solution induced a reversible increase in pH_i value under the HCO_3^-/CO_2-buffered condition, while it had hardly any effect on pH_i under the Hepes-buffered condition. The K^+-induced pH_i changes were partially suppressed by removing Cl^- from the superfusion solution. These results, together with the reported findings of mouse slc26a3, slc26a6 and AE2 in heterologously expressed systems, suggest the possibility that these three exchangers may all be functionally expressed in mouse ileal villous cells.

Villous enterocytes in the small intestine demonstrate plasma membrane Cl^-/HCO_3^-- exchange activity which is believed to be involved in absorbing NaCl by coupling with Na^+/H^+ exchange, in protecting mucosa by mediating HCO_3^- secretion and in regulating the intracellular pH value (5, 10, 21, 30, 31). Three kinds of membrane Cl^-/HCO_3^- exchangers have recently been identified in the mammalian small intestine, *i.e.*, SLC26A6 (alias PAT1) , SLC26A3 (alias DRA) and AE2 (alias SLC4A2) (11, 13, 15, 17, 20, 22, 23, 26, 28, 33). Both SLC26A6 and SLC26A3 are mainly expressed in the apical membrane, and are assumed to be responsible for Cl^- absorption and HCO_3^- secretion (11, 32). On the other hand, AE2 is expressed mainly in the basolateral membrane and probably exercises the housekeeping function of intracellular pH regulation (1, 2). The transport and pharmacological characteristics of these three Cl^-/HCO_3^- exchangers have been partially revealed by using the heterologously expressed systems (6, 7, 9, 12, 14, 17, 18, 27, 34).

The objective of this present study is to examine whether SLC26A6, SLC26A3 and AE2 actually operate in the native enterocyte. To do this, we evaluated the Cl^-/HCO_3^-- exchange activity in isolated mouse villi by microfluorometric intracellular pH (pH_i) measurement (8). We examined the anion selectivity, inhibitor sensitivity, and electrogenicity of Cl^-/HCO_3^- exchange to compare with the reported

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observations for mouse slc26a6, slc26a3, and AE2 by using heterologously expressed systems. We specifically selected the ileum, since this segment has been relatively disregarded in comparison with the proximal small intestine (11, 29, 32, 33) and the proximal and distal small intestinal actions may not be identical.

MATERIALS AND METHODS

Tissue preparation. The present experiments were performed under the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences recommended by the Physiological Society of Japan. Male mice (ddy; Japan SLC, Shizuoka, Japan) weighing 35–40 g were acclimatized on a standard diet, with food and water being provided *ad libitum*. The animals were killed by dislocation of the cervical vertebrae. A segment of the distal ileum was excised, opened and kept in an oxygenated Hepes-buffered solution. Several tips of villi were cut from each ileal segment by microdissection scissors under a stereomicroscope while the intestinal segment was pinned with the mucosal side upward, as described previously for the guinea-pig ileum (8). These were subsequently used for measuring the pH_i value and for an RT-PCR analysis.

RT-PCR analysis. Total RNA was extracted from the villus tips by using an RNeasy® micro kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The RNA samples were then subjected to the RT-PCR reaction with a OneStep RT-PCR kit (Qiagen) according to the manufacturer's instructions. The primers used in the RT-PCR analysis were as follows: mouse slc26a6, forward primer 5'-TGTACTTCGCCAATGCTGAGC-3' and reverse primer 5'-GTCCAGGATGAGGTATGGA-3' (Genbank Accession No. NM134420); mouse slc26a3, forward primer 5'-TTCCTGGACCATCTCAA AGG-3' and reverse primer 5'-TGTAACGAC AACTCCCACCA-3' (Genbank Accession No. NM021353); mouse AE2, forward primer 5'-ATT GAGGAAGGGGAGGAAGA-3' and reverse primer 5'-GTCTTCGCTCCTGAAGGTTG-3' (Genbank Accession No. NM009207); mouse GAPDH; forward primer 5'-GTGTTCCTACCCCCAATGTG-3' and reverse primer 5'-TGTGAGGGAGATGCT CAGTG-3' (Genbank Accession No. NM008084). The amplification conditions were as follows: reverse transcription for 30 min at 50°C was followed by the initial PCR activation step for 15 min at 95°C, denaturation for 45 s at 94°C, annealing for 45 s at 55°C, and extension for 1 min at 72°C; 35 PCR cycles were performed. The PCR products were separated in 2% agarose gel with a Tris/acetic acid/EDTA buffer, and then detected by ethidium bromide.

Measurement of pH_i. The villus tip preparation was washed several times by centrifugation, before being re-suspended for 10 min in the dark at room temperature (20–24°C) in a Hepes-buffered solution containing 10 μM of 2'7'-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM) which had first been dissolved to a concentration of 10 mM in dimethyl sulfoxide. After loading the dye, the villus tip preparation was washed with the Hepes-buffered solution and subjected to intracellular pH measurement (8). The suspension of dye-loaded villi was first seeded on a specially designed perfusion vessel, which had been coated with a polylysine cell adhesive (Poly-L-Lysine Solution, Sigma), before the vessel was centrifuged at 200 g for 5 min at room temperature to fix the villus preparation. The vessel was then placed on the stage of an inverted microscope (TMD, Nikon, Tokyo, Japan) equipped with a CAM-230 dual-wavelength fluorometric system (Japan Spectroscopic, Tokyo, Japan), before being perfused at 6 mL/min with a gas-equilibrated solution and maintained at 34°C by pre-warming the inflow line. The whole area of a selected villus tip was alternately illuminated at 440 and 500 nm for 50 ms by a chopper at 1000 Hz, and the fluorescence was measured at 520–570 nm through a band-pass filter. The pH_i value was calculated from the mean fluorescence ratio (F500/F440) every 2 s. This complete procedure was controlled by a computer (Macintosh LC) which was equipped with a Lab View 2 data acquisition and analysis system (National Instruments, Houston, TX, USA). There was negligible autofluorescence from the villus tip.

The pH_i level was calibrated *in situ* by the nigericin-K^+ method. Dye-loaded villus tips were incubated with nigericin (20 μM) for 5 min, and then with a high-K^+ perfusion solution of various pH values (KCl, 130 mM; NaCl, 10 mM; $CaCl_2$, 1.5 mM; $MgCl_2$, 1.0 mM; Hepes or Mes, 10 mM; pH was adjusted with Tris at 37°C) to determine the relationship between the fluorescence ratio (F500/F440) and pH_i value. Calibration curves were obtained over a pH range of 6–8.

The Cl^-/HCO_3^--exchange activity was determined from the pH_i change induced by removing Cl^- from the perfusing solution. We performed the Cl^--remov-

al challenge two or three times on the same tissue, but under different experimental conditions. The results were analyzed after assuming that the time-dependent change in the response to Cl^- removal was negligible. This assumption was confirmed to be valid by the response not being significantly different when the first and third challenges were performed under control conditions (*cf.* Figs. 3 and 6).

Solutions and chemicals. The Hepes-buffered standard solution had the following composition (mM): NaCl, 110; Na-gluconate, 25; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose 10; Hepes, 15; the pH value adjusted to 7.4 with NaOH and the solution was gassed with 100% O_2. The CO_2/HCO_3^--buffered standard solution had the following composition (mM): NaCl, 110; $NaHCO_3$, 25; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; Hepes, 15; the solution was gassed with 95% O_2-5% CO_2. The Cl^--free solution was prepared by equimolar substitution of Cl^- with gluconate and by increasing Ca-$(gluconate)_2$ concentration to 5 mM. The high-K^+ solution had the following composition (mM): NaCl, 55; $NaHCO_3$, 25; KCl, 60; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; Hepes, 15. The normal K^+ solution for the high-K^+ experiment had the following composition (mM): NaCl, 55; $NaHCO_3$, 25; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; Hepes, 15; N-methyl-D-glucamine-Cl, 55. The normal K^+, Cl^--free solution had the following composition (mM): Na-gluconate, 55; $NaHCO_3$, 25; K-gluconate, 5; Ca-$(gluconate)_2$, 5; Mg-$(gluconate)_2$, 1; glucose, 10; Hepes, 15; mannitol, 110. The high K^+, Cl^--free solution had the following composition (mM): Na-gluconate, 55; $NaHCO_3$, 25; K-gluconate, 60; Ca-$(gluconate)_2$, 5; Mg-$(gluconate)_2$, 1; glucose, 10; Hepes, 15. The pH value of each of these solutions was adjusted to 7.4.

We obtained nigericin, 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS), and niflumic acid from Sigma (St. Louis, MO, USA), and BCECF-AM from Molecular Probes (Eugene, OR, USA). 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) was obtained from RBI (Natick, MA, USA), and tenidap was generously presented by Pfizer (Groton, CT, USA).

Statistical analysis. Each experimental value is given as the mean ± SE (n = no. of animals). Two-group comparisons were analysed by a paired Student's two-tailed *t*-test. A probability value of < 0.05 has been considered significant in all instances.

RESULTS

Expression of Cl^-/HCO_3^- exchangers in mouse ileal villi

We first performed RT-PCR to detect the expression of mouse slc26a6, slc26a3 and AE2 mRNAs in the mouse ileal villus preparation which was used later for the pH_i measurements. The primers were designed to obtain the expected PCR products of 371 bp, 324 bp and 349 bp for slc26a6, slc26a3 and AE2, respectively. As shown in Fig. 1, the expected-size PCR fragment was amplified for each exchanger, demonstrating that mRNAs of slc26a6, slc26a3 and AE2 had all been expressed in the ileal villi.

Cl^-/HCO_3^--exchange activity

We then examined the Cl^-/HCO_3^--exchange activity in the ileal villous cells by microfluorometric pH_i measurement, using the pH-sensitive dye BCECF. As shown in Fig. 2, the pH_i value was rapidly increased by Cl^- removal ($\Delta pH_i/min = 0.24 \pm 0.03$, $n = 5$) and then restored to the baseline level by Cl^- re-addition ($\Delta pH_i/min = -0.27 \pm 0.03$) when a villus was superfused with the HCO_3^-/CO_2-buffered solution. The magunitude of the increase in pH_i was 0.13 ± 0.02. In contrast, the pH_i increase upon Cl^- removal was greatly diminished when superfused with the HCO_3^-/CO_2-free, Hepes-buffered solution. This indicates that the ileal villous cells had Cl^-/HCO_3^--exchange activity, but little Cl^-/OH^--exchange activity. The baseline pH_i value was 7.05 ± 0.05 ($n = 5$) in the HCO_3^-/CO_2-buffered solution and 7.21 ± 0.02 ($n = 5$) in the Hepes-buffered solution, the former being significantly lower than the latter.

Anion selectivity of the Cl^-/HCO_3^- exchanger

We next examined the other anions, *i.e.*, Br^-, I^-, F^-,

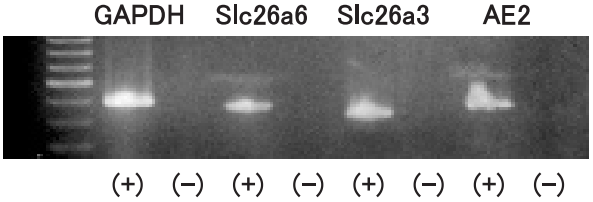

Fig. 1 RT-PCR analysis of Cl^-/HCO_3^- exchanger mRNA expressed in the mouse ileal villus tips. The amplification products were size-fractionated on agarose gel and stained with ethidium bromide. The sizes of the expected products are as follows (in bp): GAPDH, 397; mouse slc26a6; 371; mouse slc26a3, 324; mouse AE2, 349. (+) and (–) respectively show the results with and without the RT treatment. The standard (left column) is marked in 100 bp with the thickest one being 500 bp.

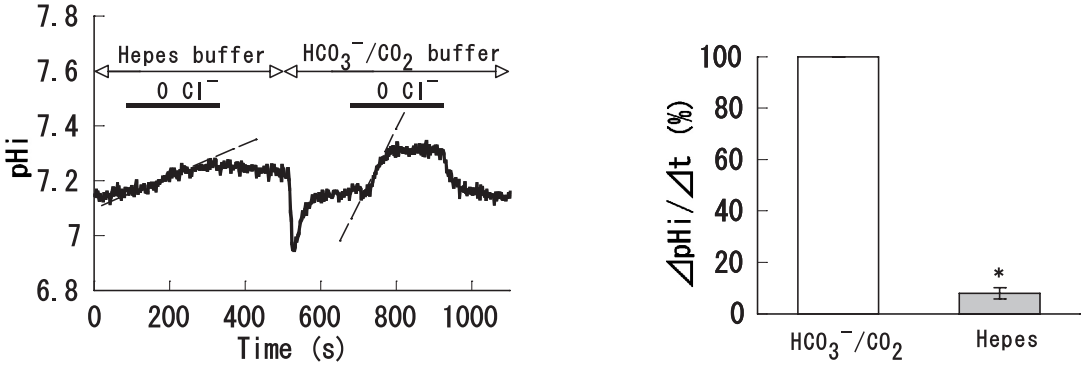

Fig. 2 pH_i changes induced by Cl^- removal in the presence and absence of HCO_3^-/CO_2 in the superfusion solution. Left panel: A typical recording. The pH_i change for a single villus loaded with BCECF-AM was recorded. Gluconate was substituted for Cl^- in the Cl^--free solution. Right panel: the rates of increase in pH_i ($\Delta pH_i/\Delta t$) upon Cl^- removal were compared in the HCO_3^-/CO_2-buffered solution and in the HCO_3^-/CO_2-free, Hepes-buffered solution. *$p < 0.05$, $n = 5$.

NO_3^-, and SO_4^{2-}, for their ability to induce HCO_3^- efflux. As shown in Figs. 3A-E, all these anions caused a pH_i decrease when added in the absence of Cl^-, indicating that, like Cl^- they could activate HCO_3^- efflux. The rate of pH_i decrease induced by each of these anions was significantly lower than that induced by Cl^- (Fig. 3F). The steady pH_i level after adding F^- or NO_3^- was not significantly different from the baseline pH_i; subsequent switching to the Cl^--containing solution did not affect the pH_i value (Figs. 3C, D and G). On the other hand, the steady pH_i level after adding Br^-, I^-, or SO_4^{2-} was significantly higher than the baseline level. Subsequent switching to the Cl^--containing solution further decreased pH_i to a level not different from the baseline (Figs. 3A, B, E and G).

We then tried to confirm the presence of SO_4^{2-}/HCO_3^--exchange activity, since mouse slc26a6 has been shown to transport SO_4^{2-} (7, 12, 35). In this experiment, Cl^- was absent throughout the measurement (Fig. 4). The pH_i value was increased when SO_4^{2-} was removed and restored to the original level when SO_4^{2-} was re-administered. The response upon SO_4^{2-} removal was more prominent in the HCO_3^-/CO_2-buffered solution than in the HCO_3^-/CO_2-free, Hepes-buffered solution, confirming the presence of SO_4^{2-}/HCO_3^-- exchange activity.

Effects of inhibitors on Cl^-/HCO_3^- exchange

In the next series of experiments, we studied the effect on the Cl^-/HCO_3^--exchange activity of several anion transport inhibitors (4). DIDS at a concentration of 30 μM slightly but significantly suppressed the rate of pH_i increase upon Cl^- removal (about 20%), although the rate of pH_i recovery upon Cl^- re-addition was not significantly affected by DIDS (Fig. 5A). DIDS did not noticeably affect the baseline pH_i level. Unexpectedly, DIDS at a concentration up to 100 μM did not significantly suppress the rate of pH_i decrease induced by Cl^- re-addition (Fig. 5B).

Tenidap (10 μM) and niflumic acid (30 μM), which have been reported to inhibit slc26a3 (24), significantly inhibited the pH_i increase upon Cl^- removal by 30–40% (Fig. 6). NPPB (30 μM), which has been reported to inhibit a variety of anion transporters, including the cystic fibrosis transmembrane conductance regulator (CFTR), significantly suppressed the pH_i increase upon Cl^- removal by about 20%.

Effect of the high-K^+ solution

Some authors have presented evidence for Cl^-/HCO_3^- exchange mediated by slc26a6 and slc26a3 being electrogenic (27, 35), so we examined the effect on the Cl^-/HCO_3^--exchange activity of depolarizing the membrane potential by increasing the extracellular K^+ concentration (Fig. 7A). Increasing the K^+ concentration from 5 mM (normal K^+) to 60 mM (high K^+) caused a reversible increase in the baseline pH_i value under the HCO_3^-/CO_2-buffered condition, while it had hardly any effect on the baseline pH_i value under the HCO_3^-/CO_2-free, Hepes-buffered condition, indicating that membrane depolarization induced HCO_3^- uptake and that membrane hyperpolarization stimulated HCO_3^- release. We then examined the Cl^- dependence of the membrane hyperpolarization-induced HCO_3^- release (Fig. 7B). In the absence of Cl^-, the rate of pH_i decrease induced by switching from the high-K^+ to normal-K^+ solution was decreased, but not abolished. Therefore, there were at least two membrane

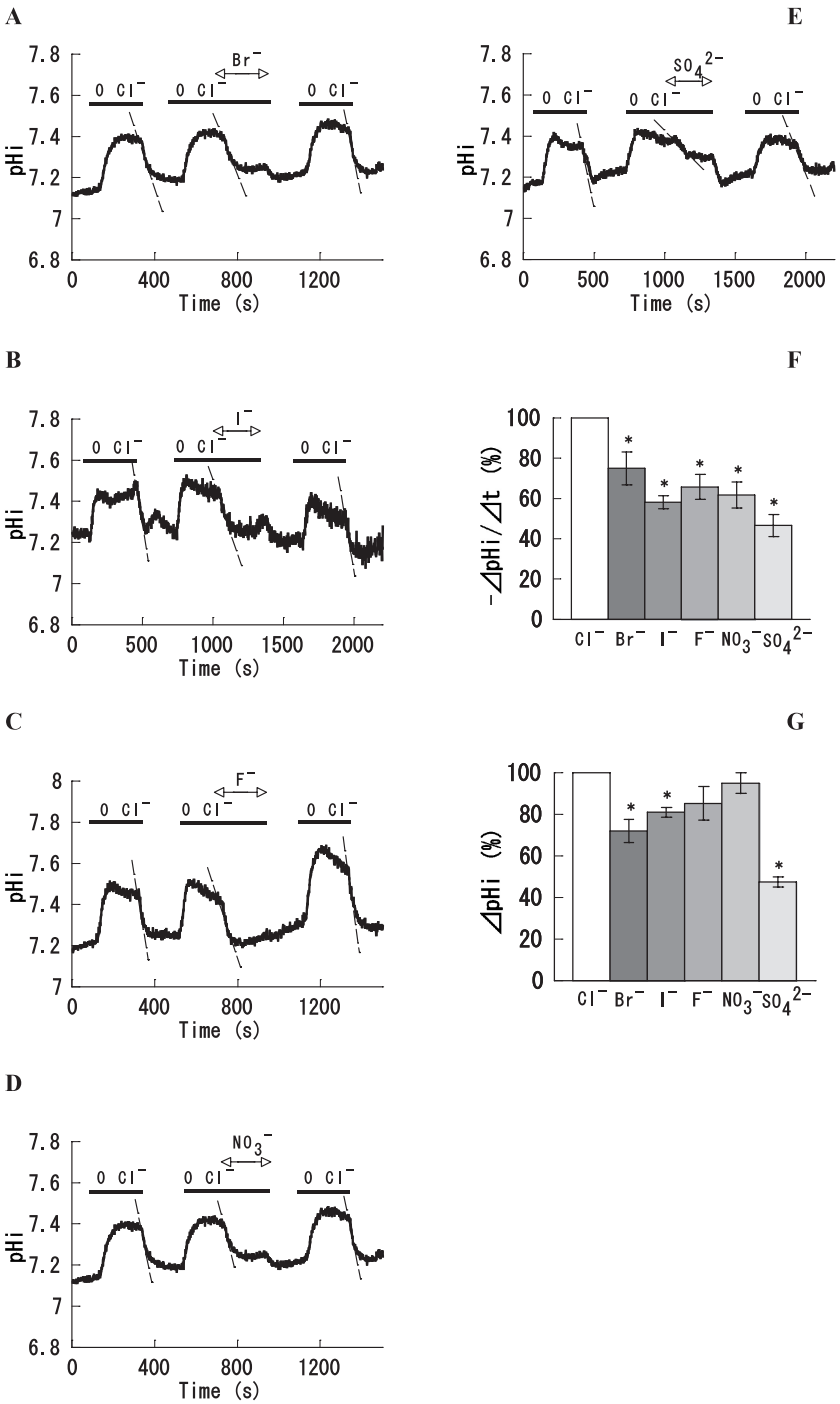

Fig. 3 Effects of various anions on the HCO_3^- efflux. The pH_i decrease induced by Br^-, I^-, F^-, NO_3^- or SO_4^{2-} was determined in the absence of Cl^-, and compared with that induced by Cl^-. A-E: Typical recordings. The Cl^--removal and re-addition challenge was performed three times on each villus. In the second challenge, the normal Cl^--free solution was first switched to the Cl^--free solution containing a test anion, and then to the Cl^--containing solution. F: the rate of pH_i recovery induced by each test anion was compared with that induced by Cl^- (the mean rate of the Cl^--induced pH^- recovery obtained from the first and final challenges). *$p < 0.05$, $n = 4–6$. G: the magnitude of the pH_i decrease induced by each test anion was compared with that induced by Cl^- (the mean value of the first and final challenges). *$p < 0.05$, $n = 4–6$. The rate and magnitude of the pH_i decrease in the final Cl^--induced response were not significantly different from those of the first Cl^--induced response without regard to the tested anions (data not shown). Br^-, I^-, F^- and NO_3^- solutions were prepared by substituting 110 mM Na^+ gluconate in the Cl^--free solution with 110 mM of each anion (Na salt), while the SO_4^{2-} solution was prepared by substituting with 55 mM Na_2SO_4 and 55 mM mannitol.

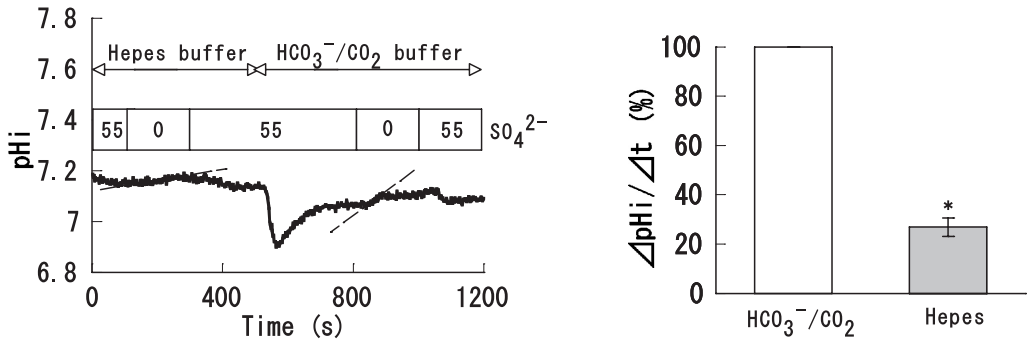

Fig. 4 SO_4^{2-} transport in exchange for HCO_3^-. Typical recording (left panel) and a summery (right panel). Cl^- was absent throughout the experiment. After the pH_i level had been stabilized in the solution containing 55 mM SO_4^{2-}, the tissue was challenged first with the SO_4^{2-}-free solution in the Hepes-buffered solution and then challenged again in the HCO_3^-/CO_2-buffered solution. The rate of increase in the pH_i value upon SO_4^{2-} removal was compared between the presence and absence of HCO_3^-/CO_2. *$p < 0.05$, $n = 4$.

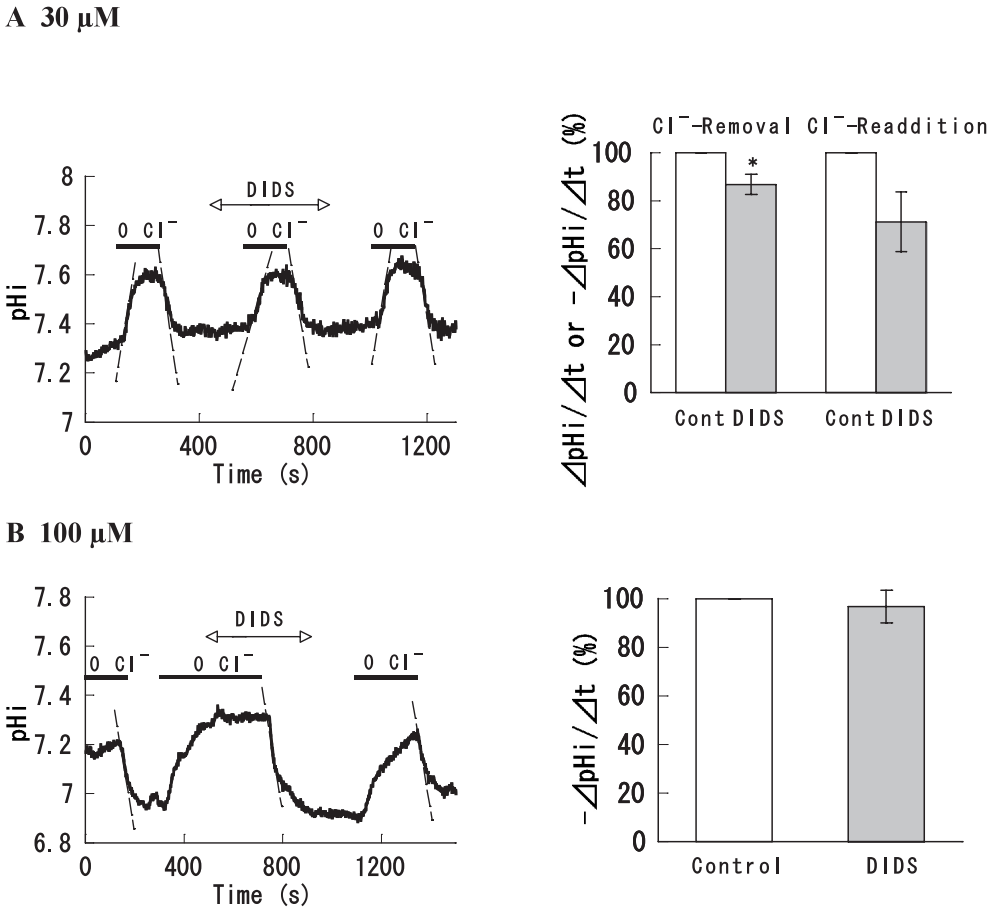

Fig. 5 Effect of DIDS on the Cl^-/HCO_3^--exchange activity. A: Typical recording (left panel) and summary (right panel) of the effect of 30 μM DIDS. Each tissue was challenged three times by Cl^- removal, the first and final challenges being without DIDS and the second challenge being with DIDS. The rates of pH_i increase and decrease induced by the Cl^--removal challenge in the presence of 30 μM DIDS (second challenge) was compared with the mean of the first and final control challenges, which were not significantly different from each other (data not shown). *$p < 0.05$, $n = 4$. B: Typical recording (left panel) and summary (right panel) of the effect 100 μM DIDS. The rate of pH_i decrease in the presence of DIDS was compared with only that observed in the first control Cl^--removal challenge, since the rate observed in the third control challenge appeared to be slightly lower than the rate in the first challenge. This was probably due to slow and irreversible inhibition by a high concentration of DIDS (4). $n = 4$.

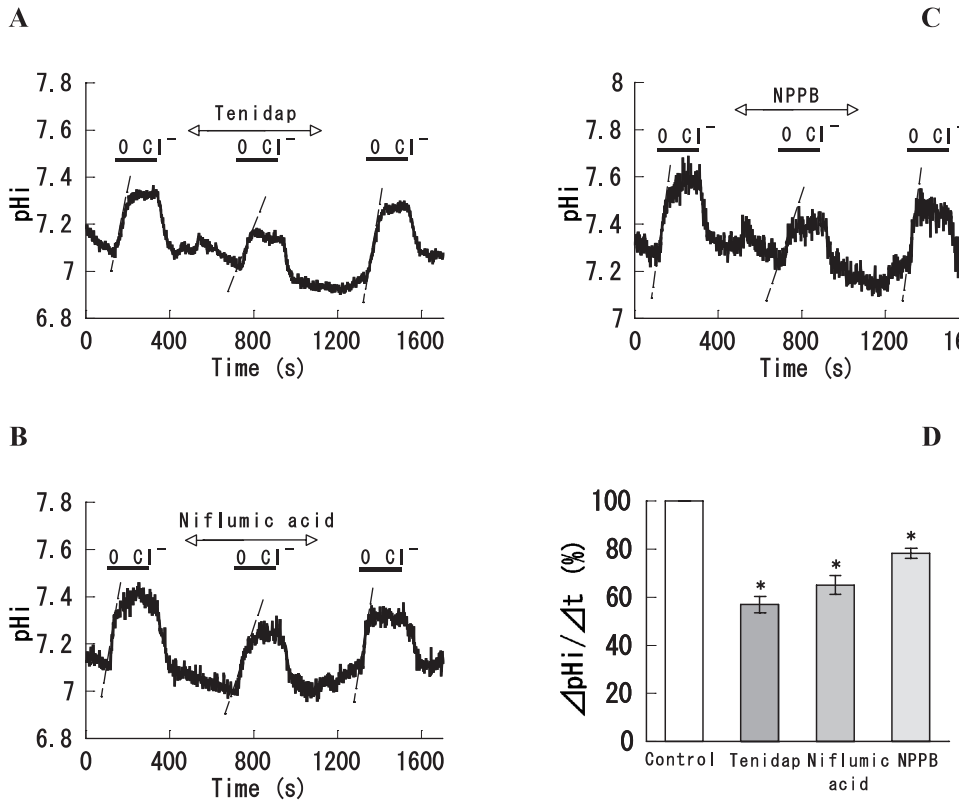

Fig. 6 Effects of tenidap, niflumic acid and NPPB on the Cl^-/HCO_3^--exchange activity. A-C: Typical recordings. D: Summary of the effects of 10 μM tenidap, 30 μM niflumic acid and 30 μM NPPB. Since the inhibitors caused a decrease in the baseline pH_i value (tenidap, 7.29 ± 0.06 to 7.20 ± 0.08, $p = 0.165$; niflumic acid, 7.12 ± 0.01 to 7.04 ± 0.02, $p < 0.05$; NPPB, 7.13 ± 0.06 to 7.05 ± 0.16, $p < 0.05$), the slopes of the pH_i increase upon Cl^- removal were determined at the same pH_i level for each tissue. Control values were determined as the mean of the rates obtained in the first and final Cl^--removal challenges, these not being significantly different from each other without regard to the tested inhibitors (data not shown). *$p < 0.05$, $n = 5$.

hyperpolarization-induced HCO_3^- efflux pathways in operation: one was Cl^--dependent and the other was Cl^--independent.

DISCUSSION

We initially showed in the present study by RT-PCR that mRNAs for slc26a6, slc26a3 and AE2 were are all expressed in the villus of the mouse ileum, confirming the results of previous studies showing that mRNA for these three anion exchangers and proteins for slc26a6 and AE2 were expressed in the mouse small intestine (1, 6, 9, 20, 32). We then demonstrated the presence of Cl^-/HCO_3^--exchange activity in the same preparation. Although previous studies have shown the presence of Cl^-/HCO_3^--exchange activity in villous epithelial cells of the mouse proximal small intestine (duodenum) (29, 33), it remains to be proved whether Cl^-/HCO_3^--exchange activity also occurs in the mouse ileum.

The present results suggest that at least some of the anion exchangers present in the ileal villi can transport not only Cl^-, but also Br^-, I^-, F^-, SO_4^{2-} and NO_3^- in exchange for HCO_3^- (Figs. 3 and 4). Mouse slc26a6 has been reported to transport Cl^-, NO_3^-, Br^-, I^- and SO_4^{2-} in a heterologously expressed system (7, 12, 35). Mouse AE2 has been shown to transport Cl^-, NO_3^- and Br^-, but not I^-, and SO_4^{2-} transport is not known (9). Mouse slc26a3 has been reported to transport NO_3^- in exchange for HCO_3^-, but the transport of other anions is not known (26). No matter which transporters are involved, the transport of Cl^-, Br^-, I^-, F^- and SO_4^{2-} across the enterocyte membrane could be responsible for the intestinal absorption of these anions as essential nutrients. This notion may also be applied to the transport of NO_3^-, because NO_3^- is contained in food and has been postulated to be absorbed in the small intestine (19).

We have shown that DIDS at a concentration of 30 μM slightly suppressed (by about 20%) the Cl^-/

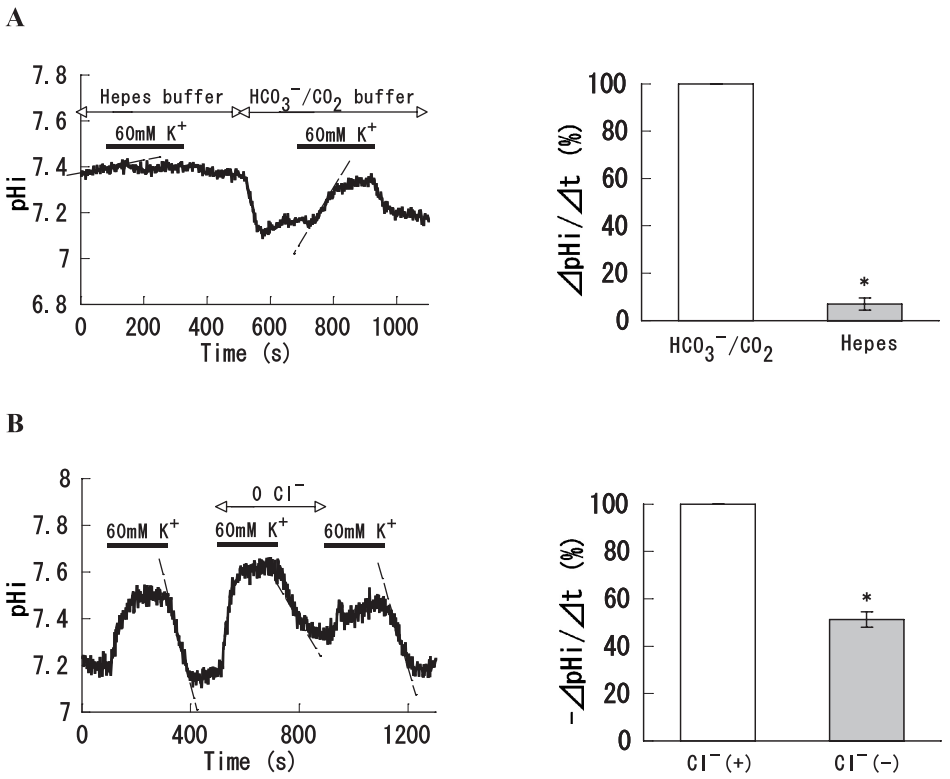

Fig. 7 Effect of the high-K^+ solution on the Cl^-/HCO_3^--exchange activity. A: Typical recording (left panel) and summary (right panel) of the K^+-induced pH_i change. The rates of pH_i increase induced by switching from the 5 mM K^+- to 60 mM K-containing solution were compared in the Hepes-buffered and HCO_3^-/CO_2-buffered solutions. *$p < 0.05$, $n = 4$. B: Typical recording (left panel) and summary (right panel) of the Cl^- dependence of the K^+-induced pH_i change in the HCO_3^-/CO_2-buffered solution. The rates of pH_i decrease induced by changing the K^+ concentration in the perfusate from 60 mM to 5 mM were compared in the presence and absence of Cl^-. Since Cl^- removal shifted the pH_i value to a higher level, the rates of pH_i decrease were compared at the same pH_i level for each tissue. The value in the presence of Cl^- was the mean of the first and third measurements which were not significantly different from each other (data not shown). *$p < 0.05$, $n = 10$.

HCO_3^- exchange activity, but that increasing the DIDS concentration up to 100 μM did not further suppressed it (Fig. 5). Mouse slc26a3 has been shown to be inhibited by 1 mM DIDS by only 24% in a heterologously expressed system, indicating that its relative resistance to DIDS (20). In contrast, Cl^-/HCO_3^- exchange mediated by mouse slc26a6 was inhibited by 5 μM DIDS by about 90% (7, 12, 27). AE2 has also been reported to be sensitive to DIDS: the Cl^- uptake mediated by mouse AE2 and the Cl^-/HCO_3^- exchange mediated by rat AE2 were inhibited by DIDS with an ED_{50} of less than about 20 μM (9, 18). It is not clear why 100 μM DIDS did not affect the Cl^-/HCO_3^-- exchange activity (Fig. 5B), although a possible explanation is that a high concentration of DIDS had a stimulatory effect on the exchange activity in addition to the inhibitory effect, and that both effects cancelled each other.

We found that niflumic acid, tenidap and NPPB each partially inhibited Cl^-/HCO_3^- exchange. Niflumic acid and tenidap have been reported to inhibit mouse slc26a6 (7) and human SLC26A3 (6). However, the sensitivity of mouse slc26a6 to tenidap and NPPB, and of mouse slc26a3 and AE2 to niflumic acid, tenidap and NPPB is not known.

We found that increasing or decreasing the extracellular K^+ concentration respectively induced an uptake or release of HCO_3^-. Since the membrane K^+ channels are ubiquitously expressed, respectively increasing or decreasing the extracellular K^+ concentration may well depolarize or hyperpolarize the membrane potential, thereby driving HCO_3^- transport if it occurs through an electrogenic pathway. A portion of this K^+-induced HCO_3^- transport was Cl^- dependent (Fig. 7). The Cl^--dependent, electrogenic HCO_3^- transport pathway may be mediated by slc26a6, because mouse and human slc26a6 has been suggested to mediate electrogenic Cl^-/HCO_3^- exchange with stoichiometry of $HCO_3^- > Cl^-$ (14, 27, 35), although some authors have provided evi-

dence against the electrogenicity of SLC26A6 (7). Slc26a3 may also be electrogenic, but could not mediate this K^+-induced HCO_3^- transport since it has been suggested to operate with stoichiometry of $HCO_3^- < Cl^-$ (6, 14, 16, 27). AE2 is probably electroneutral (26), excluding the possibility that this is involved in mediating K^+-induced HCO_3^- transport.

One possible mechanism to explain Cl^--independent, K^+-induced HCO_3^- transport is an HCO_3^--permeable channel. CFTR is one candidate for such a channel and has been reported to be present in mouse villous enterocytes of the proximal small intestine (3), while also being known to permeate HCO_3^- (24, 25). It is also possible that Cl^--independent, K^+-induced HCO_3^- transport is mediated by the K^+-HCO_3^- co-transporter. Such a co-transporter has previously been suggested to be present in squid axon (37) and in the medullary thick ascending limb of rat kidney (34), although its molecular identity remains to be determined. Since K^+-induced HCO_3^- transport has also been observed in the villous cells of the mouse proximal small intestine (29) and guinea pig distal small intestine (Hayashi, unpublished observation), it may be a widely-distributed phenomenon and, therefore, an important issue to be addressed further.

In summary, it is at least possible that slc26a6, slc26a3 and AE2 are all involved in the Cl^-/HCO_3^- exchange activity in mouse ileal villous cells. slc26a6 in particular may be involved, since SO_4^{2-}/HCO_3^- exchange activity was demonstrated. The inhibition of Cl^-/HCO_3^- exchange by niflumic acid and DIDS also agrees with the functional expression of slc26a6. The possible presence of electrogenic Cl^-/HCO_3^- exchange with stoichiometry of $Cl^- < HCO_3^-$ could also support the involvement of slc26a6, although the relatively small inhibition of Cl^-/HCO_3^- exchange by DIDS may exclude any substantial involvement of slc26a6. slc26a3 may also be implicated in the Cl^-/HCO_3^- exchange activity, since a substantial portion of this exchange was DIDS-insensitive, while being inhibited by tenidap and niflumic acid. AE2 may also mediate Cl^-/HCO_3^- exchange activity judging from the inhibition by DIDS. We have considered mainly the studies on mouse exchangers in this discussion, and not on human orthologues, since important differences have been demonstrated for SLC26A6 between the mouse and human (7). Several other Cl^-/HCO_3^- exchangers, *i.e.*, AE3 and AE4, have been suggested to be present in intestinal epithelial cells (2, 15, 36), and thus could also be responsible for the Cl^-/HCO_3^--exchange activity in the mouse ileum that was observed here. In addition, it is by no means inevitable that the HCO_3^- transport induced by Cl^- removal would be due to Cl^-/HCO_3^- exchangers, because it could also be the result of indirect, electrical coupling when Cl^- and HCO_3^- are both transported through the anion channels (3, 24, 25).

Previous studies on villous epithelial cells from the mouse proximal small intestine (duodenum) have demonstrated the major role of slc26a6 in the Cl^-/HCO_3^- exchange activity (29, 33). The proximal and distal parts of the small intestine may have distinctly different physiological functions from each other and, therefore, it would be interesting to further explore the difference in enterocyte Cl^-/HCO_3^- exchange between the proximal and distal small intestine.

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REFERENCES

1. Alper SL, Rossmann H, Wilhelm S, Stuart-Tilley AK, Shmukler BE and Seidler U (1999) Expression of AE2 anion exchanger in mouse intestine. *Am J Physiol Gastrointest Liver Physiol* **277**, G321–G332.
2. Alrefai WA, Tyagi S, Nazir TM, Barakat J, Anwar SS, Hadjiagapiou C, Bavishi D, Sahi J, Malik P, Goldstein J, Layden TJ, Ramaswamy K and Dudeja PK (2001) Human intestinal anion exchanger isoforms: expression, distribution, and membrane localization. *Biochim Biophys Acta* **1511**, 17–27.
3. Ameen N, Alexis J and Salas P (2000) Cellular localization of the cystic fibrosis transmembrane conductance regulator in mouse intestinal tract. *Histochem Cell Biol* **114**, 69–75.
4. Cabantchik ZI and Greger R (1992) Chemical probes for anion transporters of mammalian cell membranes. *Am J Physiol Cell Physiol* **262**, C803–C827.
5. Calonge ML, Molina MT and Ilundain A (1992) Cl^-/base exchange and cellular pH regulation in enterocytes isolated from chick small intestine. *Biochim Biophys Acta* **1103**, 45–50.
6. Chernova MN, Jiang L, Shmukler BE, Schweinfest CW, Blanco P, Freedman SD, Stewart AK and Alper SL (2003) Acute regulation of the SLC26A3 congenital chloride diarrhoea anion exchanger (DRA) expressed in Xenopus oocytes. *J Physiol* **549**, 3–19.
7. Chernova MN, Jiang L, Friedman DJ, Darman RB, Lohi H, Kere J, Vandorpe DH and Alper SL (2005) Functional comparison of mouse slc26a6 anion exchanger with human SLC26A6 polypeptide variants: differences in anion selectivity, regulation, and electrogenicity. *J Biol Chem* **280**, 8564–8580.
8. Hayashi H and Suzuki Y (1998) Regulation of intracellular pH during H^+-coupled oligopeptide absorption in enterocytes from guinea-pig ileum. *J Physiol* **511**, 573–586.
9. Humphreys BD, Jiang L, Chernova MN and Alper SL (1994) Functional characterization and regulation by pH of murine

AE2 anion exchanger expressed in Xenopus oocytes. *Am J Physiol Cell Physiol* **267**, C1295–C1307.
10. Ilundain A (1992) Intracellular pH regulation in intestinal and renal epithelial cells. *Comp Biochem Physiol* **101**, 413–424.
11. Jacob P, Rossmann H, Lamprecht G, Kretz A, Neff C, Lin-Wu E, Gregor M, Groneberg DA, Kere J and Seidler U (2002) Down-regulated in adenoma mediates apical Cl^-/HCO_3^- exchange in rabbit, rat, and human duodenum. *Gastroenterology* **122**, 709–724.
12. Jiang Z, Grichtchenko II, Boron WF and Aronson PS (2002) Specificity of anion exchange mediated by mouse Slc26a6. *J Biol Chem* **277**, 33963–33967.
13. Kere J, Lohi H and Hoglund P (1999) Genetic Disorders of Membrane Transport III. Congenital chloride diarrhea. *Am J Physiol* **276**, G7–G13.
14. Ko SB, Shcheynikov N, Choi JY, Luo X, Ishibashi K, Thomas PJ, Kim JY, Kim KH, Lee MG, Naruse S and Muallem S (2002) A molecular mechanism for aberrant CFTR-dependent HCO_3^- transport in cystic fibrosis. *EMBO J* **21**, 5662–5672.
15. Kudrycki KE, Newman PR and Shull GE (1990) cDNA cloning and tissue distribution of mRNAs for two proteins that are related to the band 3 Cl^-/HCO_3^- exchanger. *J Biol Chem* **265**, 462–471.
16. Lamprecht G, Baisch S, Schoenleber E and Gregor M (2005) Transport properties of the human intestinal anion exchanger DRA (down-regulated in adenoma) in transfected HEK293 cells. *Pflugers Arch-Eur J Physiol* **449**, 479–490.
17. Lecanda J, Urtasun R and Medina JF (2000) Molecular cloning and genomic organization of the mouse AE2 anion exchanger gene. *Biochem Biophys Res Commun* **276**, 117–124.
18. Lee BS, Gunn RB and Kopito RR (1991) Functional differences among nonerythroid anion exchangers expressed in a transfected human cell line. *J Biol Chem* **266**, 11448–11454.
19. McKnight GM, Duncan CW, Leifert C and Golden MH (1999) Dietary nitrate in man: friend or foe? *Br J Nutr* **81**, 349–358.
20. Melvin JE, Park K, Richardson L, Schultheis PJ and Shull GE (1999) Mouse down-regulated in adenoma (DRA) is an intestinal Cl^-/HCO_3^- exchanger and is up-regulated in colon of mice lacking the NHE3 Na^+/H^+ exchanger. *J Biol Chem* **274**, 22855–22861.
21. Montrose MH and Kere J (2001) Anion absorption in the intestine: anion transporters, short-chain fatty acids, and role of the DRA gene product. In: *Gastrointestinal Transport* (Barrett KE and Donowitz M, eds.), pp.301–328, Academic Press, San Diego.
22. Mount DB and Romero MF (2004) The SLC26 gene family of multifunctional anion exchangers. *Pflugers Arch* **447**, 710–721.
23. Petrovic S, Wang Z, Ma L, Seidler U, Forte JG, Shull GE and Soleimani M (2002) Colocalization of the apical Cl^-/HCO_3^- exchanger PAT1 and gastric H^+-K^+-ATPase in stomach parietal cells. *Am J Physiol Gastrointest Liver Physiol* **283**, G1207–G1216.
24. Poulsen JH, Fischer H, Illek B and Machen TE (1994) Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator. *Proc Natl Acad Sci USA* **91**, 5340–5344.
25. Reddy MM and Quinton PM (2003) Control of dynamic CFTR selectivity by glutamate and ATP in epithelial cells. *Nature* **423**, 756–760.
26. Romero MF, Fulton CM, and Boron WF (2004) The SLC4 family of HCO_3^- transporters. *Pflugers Arch* **447**, 495–509.
27. Shcheynikov N, Wang Y, Park M, Ko SBH, Dorwart M, Naruse S, Thomas PJ and Muallem S (2006) Coupling modes and stoichiometry of Cl^-/HCO_3^- exchange by slc26a3 and slc26a6. *J Gen Physiol* **127**, 511–524.
28. Silberg DG, Wang W, Moseley RH and Traber PG (1995) The down-regulated in adenoma (DRA) gene encodes an intestine-specific membrane sulfate transport protein. *J Biol Chem* **270**, 11897–11902.
29. Simpson JE, Gawenis LR, Walker NM, Boyle KT and Clarke LL (2005) Chloride conductance of CFTR facilitates basal Cl^-/HCO_3^- exchange in the villous epithelium of intact murine duodenum. *Am J Physiol Gastrointest Liver Physiol* **288**, G1241–G1251.
30. Sullivan SK and Field M (1991) Ion transport across mammalian small intestine. In: *Handbook of Physiology*, Section 6, the Gastrointestinal System, vol. IV, Intestinal Absorption and Secretion (Schultz SG, Field M, Frizzell RA, and Rauner BB, eds.), pp.287–301, American Physiological Society, Bethesda.
31. Sundaram U, Knickelbein RG and Dobbins JW (1991) pH regulation in ileum: Na^+-H^+ and Cl^--HCO_3^- exchange in isolated crypt and villus cells. *Am J Physiol* **260**, G440–449.
32. Wang Z, Petrovic S, Mann E and Soleimani M (2002) Identification of an apical Cl^-/HCO_3^- exchanger in the small intestine. *Am J Physiol Gastrointest Liver Physiol* **282**, G573–G579.
33. Wang Z, Wang T, Petrovic S, Tuo B, Riederer, B, Barone, S, Lorenz JN, Seidler U, Aronson PS and Soleimani M (2005) Renal and intestinal transport defects in Slc26a6-null mice. *Am J Physiol Cell Physiol* **288**, C957–C965.
34. Watts BA and Good DW (2004) An apical K^+-dependent HCO_3^- transport pathway opposes transepithelial HCO_3^- absorption in rat medullary thick ascending limb. *Am J Physiol Renal Physiol* **287**, F57–F63.
35. Xie Q, Welch R, Mercado A, Romero MF and Mount DB (2002) Molecular characterization of the murine Slc26a6 anion exchanger: functional comparison with Slc26a1. *Am J Physiol Renal Physiol* **283**, F826–F838.
36. Xu J, Barone S, Petrovic S, Wang Z, Seidler U, Riederer B, Ramaswamy K, Dudeja PK, Shull GE and Soleimani M (2003) Identification of an apical Cl^-/HCO_3^- exchanger in gastric surface mucous and duodenum villus cells. *Am J Physiol Gastrointestinal Liver Physiol* **285**, G1225–G1234.
37. Zhao J, Hogan EM, Bevensee MO and Boron WF (1995) Out-of-equilibrium CO_2/HCO_3^- solutions and their use in characterizing a new K^+/HCO_3^- cotransporter. *Nature* **374**, 636–639.