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The electroneutral Na^+:HCO_3^- cotransporter NBCn1 is a major pH_i regulator in murine duodenum

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Key points

- The mucosa of the proximal duodenum is regularly exposed to the strong acid of gastric juice, and acid damage causes peptic ulceration and bleeding.
- The epithelial cells of the duodenum protect themselves against acid-induced chemical damage by secreting bicarbonate into the lumen and by regulating their intracellular pH, but the molecular mechanisms of duodenal pH_i control are incompletely understood.
- We have found a high expression of the electroneutral sodium bicarbonate transporter Slc4a7 (NBCn1) in the basolateral membrane of duodenal villous enterocytes.
- Genetic knockout of Slc4a7 resulted in a strong defect in duodenocyte pH_i recovery from acid loads, and reduced bicarbonate secretory rates.
- This suggests that the electroneutral sodium bicarbonate cotransporter Slc4a7 is a major import mechanism for HCO_3^- from the blood into the duodenal epithelial cells. Defects in this transporter may severely endanger duodenal mucosal integrity

Abstract Duodenocyte pH_i control and HCO_3^- secretion protects the proximal duodenum against damage by gastric acid. The molecular details of duodenocyte pH control are not well understood. A selective duodenal expression (within the upper GI tract) has been reported for the electroneutral Na^+:HCO_3^- cotransporter NBCn1 (Slc4a7). We aimed to determine the role of NBCn1 and NBCe2 in duodenocyte intracellular pH regulation as well as basal and agonist-stimulated duodenal bicarbonate secretion (J_{HCO3}^-), exploiting mouse models of genetic *slc4a7* and *slc4a5* disruption. Basal and forskolin (FSK)-stimulated J_{HCO3}^- was measured by single-pass perfusion in the duodenum of *slc4a7*$^{-/-}$ and *slc4a7*$^{+/+}$ as well as *slc4a5*$^{-/-}$ and *slc4a5*$^{+/+}$ mice *in vivo*, and by pH-stat titration in isolated duodenal mucosa *in vitro*. Duodenocyte HCO_3^- uptake rates were fluorometrically assessed after acidification of intact villi and of isolated duodenocytes. *Slc4a7*$^{-/-}$ mice displayed significantly lower basal and FSK-stimulated duodenal HCO_3^- secretion than *slc4a7*$^{+/+}$ littermates *in vivo*. FSK-stimulated HCO_3^- secretion was significantly reduced in *slc4a7*$^{-/-}$ isolated duodenal mucosa. Na^+- and HCO_3^--dependent base uptake rates were significantly decreased in *slc4a7*$^{-/-}$ compared with *slc4a7*$^{+/+}$ villus duodenocytes when measured in intact villi. Carbonic anhydrase (CA)-mediated CO_2 hydration played no apparent role as a HCO_3^- supply mechanism for basal or FSK-stimulated secretion in the *slc4a7*$^{+/+}$ duodenum, but was an important alternative HCO_3^- supply mechanism in the *slc4a7*$^{-/-}$ duodenum. NBCe2 (Slc4a5) displayed markedly lower duodenal mRNA expression levels, and its disruption did not interfere with duodenal HCO_3^- secretion. The electroneutral

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Na^+:HCO_3^- cotransporter NBCn1 (*slc4a7*) is a major duodenal HCO_3^- importer that supplies HCO_3^- during basal and FSK-stimulated HCO_3^- secretion.

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Abbreviations CA, carbonic anhydrase; FSK, forskolin

Introduction

The gastric contents that are emptied into the duodenum occasionally reach pH values as low as 1, and the duodenal mucosa is required to protect itself against this acid load in order to avoid chemical damage (Allen & Flemstrom, 2005; Kaunitz & Akiba, 2006). The mechanisms for duodenal protection have been discussed extensively. On the one hand, a large body of circumstantial evidence suggests that the epithelium protects itself by secreting HCO_3^- ions into the lumen and thus neutralizing H^+ ions that diffuse through the mucus gel (reviewed in Allen & Flemstrom, 2005; Seidler & Sjöblom 2012). However, the duodenal villus tips in mice are not consistently covered by mucus gel, and Akiba and Kaunitz provided evidence in rodent duodenum *in vivo* that basolateral HCO_3^- uptake and intracellular neutralization of acid may play a major role in preventing epithelial damage in the villous region (Akiba *et al.* 2001).

As recently reviewed (Seidler *et al.* 2011; Seidler & Sjöblom, 2012), attempts to elucidate the pH_i regulatory mechanisms in duodenocytes, using isolated cell suspensions or short-term culture, provided functional evidence for the involvement of Na^+/H^+ exchangers (NHEs), Na^+/HCO_3^- cotransporters (NBCs) and even Cl^-/HCO_3^- exchangers in the pH_i recovery from an intracellular acid load, and the application of ion transport inhibitors allowed some further characterization of the involved NHE isoforms. However, Akiba *et al.* (2001) showed that in the rodent duodenum *in vivo*, a DIDS-sensitive mechanism is essential for pH_i recovery from intracellular acidification, suggesting the possible involvement of NBCs in duodenal pH_i recovery from acidification.

The first identified member of the Na^+:HCO_3^- cotransporter was the electrogenic Na^+:HCO_3^- cotransporter (NBC1, NBCe1) of the basolateral membrane in renal proximal tubules (Burnham *et al.* 1997; Romero *et al.* 1997). A splice variant of this gene with a different N-terminus and a different stochiometry (first named p(ancreatic)NBC1 (Abuladze *et al.* 1998) or d(uodenal) NBC1 (Jacob *et al.* 2000)) was found with a widespread distribution in gastrointestinal epithelia (Rossmann *et al.* 1999; Jacob *et al.* 2000; Jakab *et al.* 2011). Due to its high expression in the duodenum, this NBC isoform is generally regarded as an important duodenal HCO_3^- importer, but due to the early death of the NBCn1 knockout mice, this has never been functionally confirmed (Gawenis *et al.* 2007).

In 2000, an electroneutral Na^+:HCO_3^- cotransporter, NBCn1, was cloned (Choi *et al.* 2000). The study of its tissue distribution showed high expression levels in the vasculature but low levels in gastrointestinal (GI) organs (Abuladze *et al.* 1998), thus not suggesting a high relevance for GI ion transport. However, Praetorius and colleagues demonstrated the expression of NBCn1, in addition to NBCe1 (which is also expressed in other parts of the small intestine) in the basolateral membrane of villous duodenocytes (Praetorius *et al.* 2001). NBCn1 was found to also be expressed in human duodenum (Damkier *et al.* 2007). The expression of this transporter in a gastrointestinal epithelium with a particularly high demand for intracellular pH_i regulation as well as for protective HCO_3^- secretions raises the question of its involvement in the regulation of duodenocyte pH as well as for HCO_3^- import during duodenal alkaline secretion.

Therefore, the role of NBCn1 in duodenal pH_i regulation as well as basal and agonist-stimulated duodenal HCO_3^- secretion were studied in NBCn1-deficient (*slc4a7*$^{-/-}$) mice and their WT littermates *in vivo* and *in vitro*. We also studied the compensatory mechanisms for HCO_3^- uptake that are used by NBCn1-deficient (*slc4a7*$^{-/-}$) duodenocytes.

Methods

Chemicals and solutions

All reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany) if not indicated otherwise. Forskolin (FSK) was diluted in DMSO (10^{-2} M), stored at −20°C and added to an isotonic NaCl solution to a final concentration as indicated in the text on the same day of use.

Animals

Transcription of *slc4a7* (gene for NBCn1) was interrupted by insertion of a gene trap-cassette in a CG-rich region upstream of exon1. Details of the mouse generation and general characterization are given elsewhere (Boedkjer *et al.* 2011). Transcription of *slc4a5* (gene for NBCe2) was disrupted as described in the legend to Fig. 4. Mice were bred in the animal facilities of Hannover

Table 1. Blood gas analysis of the anaesthetized mice at the end of the experiments shown in Figs 3 and 7

Group	pH	P_{CO_2} (mmHg)	HCO_3^- (mM)	SBE	No. of mice
Slc4a7$^{+/+}$ (FSK)	7.41 ± 0.04	35.4 ± 3.5	22.8 ± 3.2	−1.5 ± 3.4	7
Slc4a7$^{-/-}$ (FSK)	7.46 ± 0.02	31.3 ± 2.9	22.0 ± 1.2	−1.7 ± 0.9	3
Slc4a7$^{+/+}$ (FSK+mtz/acz)	7.35 ± 0.02	51.2 ± 3.7	27.1 ± 0.8	1.9 ± 0.7	10
Slc4a7$^{-/-}$ (FSK+mtz/acz)	7.38 ± 0.02	45.6 ± 1.4	26.5 ± 1.1	2.0 ± 1.2	8

The last column indicates the number of mice for which analysis could be done. Not all mice used in the experiments could be analysed (frequent occupation of the blood gas analyser).

Medical School and Department of Biomedicine, Aarhus University, on a congenic C57Bl/6 background. The mice born from *slc4a7*$^{+/-}$ breeding pairs had a non-Mendelian distribution of the genotypes at birth, with less than expected *slc4a7*$^{-/-}$ pups by approximately one third. No difference in growth, vital parameters and blood acid–base status during the experimental procedure (Table 1) were observed in the *slc4a7*$^{-/-}$ mice, and the *slc4a5*$^{-/-}$ mice appeared grossly healthy up to the time that we used them to perform HCO_3^- secretion experiments. As the *slc4a5*$^{-/-}$ mice did not display a HCO_3^- secretory defect in the duodenum and the expression of NBCe2 was markedly lower than that for NBCn1 and NBCe1 (Figs 1 and 2), a full characterization of this strain was not pursued. All studies were approved by the Hannover Medical School Committee on investigations involving animals and the 'Regierungspräsidium'. Care was taken to gender-match littermates. The mice were housed under standard temperature and light conditions (12:12 h light–dark cycle; temperature, 21–22°C) and were allowed free access to food and water. Primers for genotyping *slc4a7*-deficient mice are found in Supplementary Table S1.

Measurement of mRNA expression of NBCe1, NBCn1, NBCe2 and NHE1-3

mRNA expression levels in scraped duodenal mucosa was performed using a quantitative real-time PCR protocol exactly as described before (Broere *et al.* 2009). The primer pairs are given in Supplementary Table 1.

Immunohistochemistry. Mice were fixed via the left ventricle with 3% paraformaldehyde in phosphate-buffered salt solution (PBS, in mM: 167 Na^+, 2.8 $H_2PO_4^-$, 7.2 HPO_4^{2-}; pH 7.4). The duodeni were removed, dehydrated, embedded in paraffin wax, and 2 μm sections were cut using a rotary microtome (Leica, Wetzlar, Germany). The sections were dewaxed and stepwise rehydrated. Epitopes were retrieved by boiling the sections in 10 mM Tris buffer (pH 9) with 0.5 mM EGTA, and then quenched with 50 mM NH_4Cl. Unspecific binding was blocked by 1% BSA in PBS with 0.2% gelatin and 0.05% saponin. The sections were incubated overnight at 4°C with primary antibody, diluted in 0.1% BSA in PBS with 0.3% Triton X-100 added. The anti-NBCn1 antibody was previously described and validated (Damkier *et al.* 2006). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Dako, Hamburg, Germany) were used for visualization with 3,3′-diaminobenzidine tetrahydrochloride dissolved in PBS with 0.1% H_2O_2 for 5 min in PBS. Mayer's haematoxylin was used for counterstaining, and the sections were dehydrated in graded alcohol and xylene and mounted in hydrophobic Eukitt mounting medium (Kindler, Freiburg, Germany). Microscopy was performed on a Leica DMRE bright-field microscope equipped with a Leica DM300 digital camera.

In vivo experimental protocols

The mice were anaesthetized by spontaneous inhalation of isoflurane (Abbott Germany, Wiesbaden) and the experiments performed as described (Singh *et al.* 2009), with the following modifications: a catheter was placed in the left carotid artery for continuous infusion of a solution containing (in mM) 130 Na^+, 5.4 K^+, 0.9 Ca^{2+}, 1 Mg^{2+}, 55 Cl^-, 55 CO_3^{2-}, 27 acetate, gassed with 5% CO_2–95% O_2 at a rate of 0.35 ml h^{-1}, to correct systemic acid–base and fluid balance. Blood gas analysis was performed and the values are given in Table 1. The earlier *in vivo* experiments (Figs 3 and 7) were performed with the Univentor 400 anaesthesia unit (with oxygen mixed by a separate valve to room air by hand); the later ones (Fig. 4) with the univentor 1250 unit (where oxygen is added more precisely by the system and the air is dehumidified). The role of *slc4a7* in the regulation of duodenal luminal alkalinization rate was assessed before and after intrasegmental perfusion of forskolin (FSK, 100 μM) as described (Singh *et al.* 2008). Mild respiratory alkalosis was sometimes seen; it was due to hyperventilation during anaesthesia and was not statistically different in NBCn1-deficient and WT littermates. As systemic acetazolamide causes mild acidosis, a higher rate of sodium carbonate (75 mM CO_3^{2-}, 35 mM Cl^-) was infused to keep blood pH_i above pH 7.3 (Table 1)

Ussing chamber experiments

Isolated duodenal mucosa of NBCn1-deficient mice and WT littermates were placed in Ussing chambers and secretory studies performed according to identical protocols as described before (Tuo *et al.* 2006), except that open circuit conditions were used, potential difference (PD) and electrical resistance (R_t) were continuously recorded, and the short circuit current (I_{sc}) calculated as described in Xiao *et al.* (2012).

Fluorometric pH_i measurements and determination of base uptake rates into slc4a7$^{-/-}$ and slc4a7$^{+/+}$ duodenocytes within intact villi

For measuring pH_i in isolated duodenal villi, a piece of duodenal tissue was transferred onto the cooled stage of a dissecting microscope, and individual villi were detached from the intestine and attached to a glass coverslip pre-coated with Cell-Tak adhesive (BD Biosciences, Bedford, MA, USA). Isolated individual villi were loaded for approximately 10 min with 16 μM 2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM) (Invitrogen, Darmstadt, Germany) in Ringer solution with 500 μM DTT. After being loaded, the villi were fixed on the coverslip by covering them with a clear filter, and perfused with pre-warmed (37°C) O_2-gassed (A) or O_2–CO_2-gassed (B) buffer as needed per experimental protocol (for buffer composition see Supplementary methods). Fluorometric pH_i measurements were performed as previously described (Broere *et al.* 2009), with modifications described in Chen *et al.* (2010). Calibration was performed as described for pancreatic ducts (Hegyi *et al.* 2004). Intrinsic buffering capacity (βi) of duodenal villi was determined according to Roos & Boron (1981) as described previously (Bachmann *et al.* 2003). A detailed description of the method is given in Chen *et al.* (2010), except that the buffer capacity was determined both by acidifying the villous enterocytes with the ammonium prepulse technique and then adding small concentrations of NH_4Cl and recording the rapid increase in pH_i for each increase of [NH_4Cl], and also by alkalinizing the cells by 80 mM NH_4Cl, followed by a stepwise decrease in [NH_4Cl], and recording of the change in pH_i, in the absence of Na^+ (for stepwise increase in pH_i, or Na^+ and Cl^- for stepwise decrease in pH_i). One such pH_i trace is depicted in Supplementary Fig. S1*B*, while the intrinsic as well as the total β (intrinsic and CO_2/HCO_3^--mediated β) is shown in Supplementary Fig. S1*C*. The rates of pH_i change measured in the experiments were converted to proton flux using the equation $J_{H^+} = \Delta pH/\Delta t \times \beta_{total}$.

Statistics

Data are presented as means ± SEM. The Mann–Whitney rank-sum test, Student's *t* test or, if appropriate, the ANOVA for multiple comparisons were used for statistics, and values of $P < 0.05$ were considered significant. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Results

NBCe1, NBCe2 and NHE1–3 expression is not altered in slc4a7$^{-/-}$ duodenal mucosa

Quantitative PCR analysis of mRNA expression levels for NBCe1, NBCn1 and NBCe2 in relationship to β-actin expression in the different segments of C57B/6 mouse intestine (Fig. 1*A*) revealed that NBCn1 expression is particularly high in the duodenum. The basolateral localization of NBCe1 and NBCn1 and their villus-predominant expression was determined by immunohistochemistry; the results were identical to previously published data (Praetorius *et al.* 2001) and are therefore not shown. The specificity of the NBCn1 antibody is evident from the lack of basolateral staining in the NBCn1 KO mouse (Fig. 1*B*). NBCe2 expression was low, and since basal and FSK-stimulated duodenal HCO_3^- secretion was not decreased in NBCe2 KO mice (Fig. 4*A*–*C*), further attempts to localize this transporter were not pursued. We then studied whether major changes occur in the expression of other base importers in the absence of duodenal NBCn1 expression. The mRNA expression levels for NBCe1, NBCe2 and NHE1–3 were determined in scraped duodenal mucosa, and found to be not significantly altered in the *slc4a7*$^{-/-}$ duodenal mucosa compared with WT (Fig. 2*A*). The localization of NBCe1 was also studied by immunohistochemistry in *slc4a7*$^{-/-}$ and WT mucosa. Its distribution and intensity of staining was not significantly different in five pairs of KO and WT mice (Fig. 2*B*).

Basal and FSK-activated HCO_3^- secretion is reduced in the duodenum of anaesthetized *slc4a7*$^{-/-}$ mice

In the duodenum of anaesthetized mice, basal secretory rates were significantly lower in the *slc4a7*$^{-/-}$ than in *slc4a7*$^{+/+}$ mice (Fig. 3*A* and *B*). Perfusion of the lumen with FSK (10^{-4} M) elicited a HCO_3^- secretory response that was significantly lower in absolute HCO_3^- secretory rate (Fig. 3*A*) but not in per cent of basal rate in *slc4a7*$^{-/-}$ *vs.* *slc4a7*$^{+/+}$ duodenum (Fig. 3*C*).

No significant difference in the basal or FSK-stimulated J_{HCO3^-} was found in the duodenum of *slc4a5*$^{-/-}$ compared with *slc4a5*$^{+/+}$ anaesthetized mice, suggesting a minor role of this bicarbonate transporter in duodenal bicarbonate secretion (Fig. 4*A*–*C*).

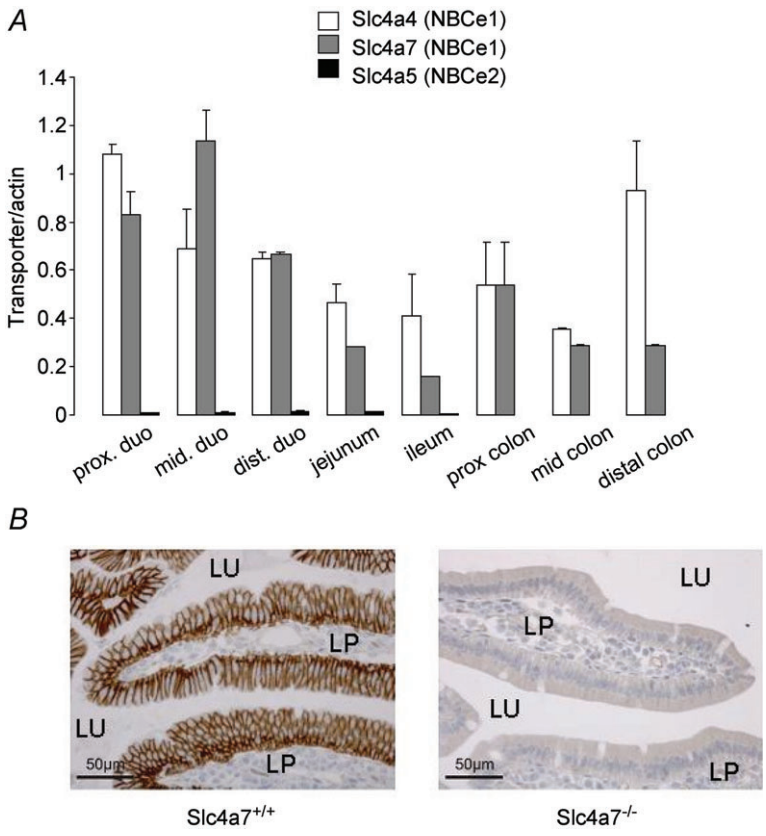

Figure 1. mRNA expression for NBCe1, NBCn1 and NBCe2 in the mucosa from different intestinal segments of C57B/6 mice, and duodenocyte basolateral NBCn1 immunostaining in WT and KO mice
A, the bars show the mRNA expression levels for the three NBCs in different segments of the GI tract, in relation to ß-actin as a control gene. NBCn1 is expressed in similar levels to that of NBCe1 in the duodenum. NBCe2 mRNA expression levels are low. $n = 6$. *B*, strong immunohistochemical staining of the basolateral membrane of the villous duodenocytes was completely absent in the *slc4a7*$^{-/-}$ duodenum. Representative staining for 5 examined pairs of mice. LU lumen, LP lamina propria.

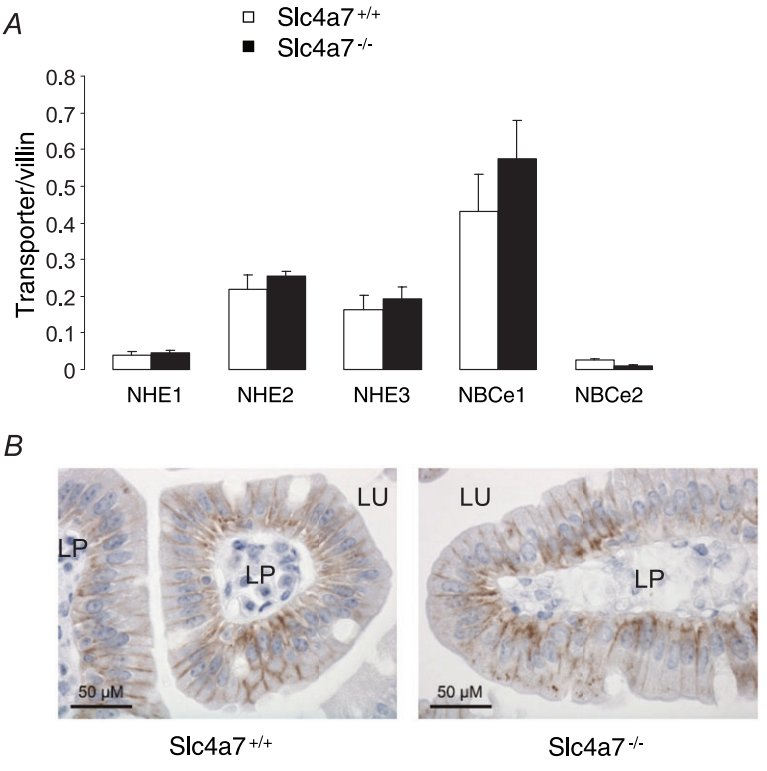

Figure 2. NHE1–3, and NBCe1 and NBCe2 mRNA expression in *slc4a7*$^{-/-}$ and *slc4a7*$^{+/+}$ duodenal mucosa
A, mRNA expression levels of NHE1–3 and of NBCe1 and NBCe2 in *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ duodenal mucosa. qPCR was performed with epithelial-specific villin as the control gene, and the respective expression level of the gene of interest was normalized to WT level. No significant change in expression level was found for the studied acid–base transporters in the absence of NBCn1 ($n = 6$). *B*, NBCe1 immunostaining in *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ duodenal mucosa. The images are representative for 5 mice examined. No difference was observed in intensity or localization of NBCe1 in *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ duodenal mucosa. LU lumen, LP lamina propria

Basal and FSK-stimulated HCO_3^- secretory rates are reduced in the isolated duodenal mucosa of *slc4a7*$^{-/-}$ mice

In muscle-stripped duodenal mucosa from *slc4a7*$^{-/-}$ and *slc4a7*$^{+/+}$ mice, basal HCO_3^- secretory rates were not significantly different (Fig. 5*A*), but FSK-stimulated HCO_3^- secretory response was significantly diminished (Fig. 5*A* and *B*). Figure 5*C* and *D* show the calculated short-circuit currents (from the continuously recorded R_t and PD) and electrical resistance (R_t) of *slc4a7*$^{-/-}$ and WT mucosa, with no difference in the results.

Steady-state pH_i and Na^+-dependent pH_i recovery in enterocytes of intact villous tips

We next assessed the effect of NBCn1 ablation on the ability of the villus enterocytes to regulate pH_i. Figure 6*A* shows a BCECF-loaded villus with the regions of interest (ROIs). Steady-state pH_i of isolated C57B/6 duodenal villi was found to be higher in the absence than in the presence of CO_2/HCO_3^-, indicating that in the absence of transepithelial gradients for pH_i and HCO_3^-, the effect of HCO_3^- efflux from the duodenocyte may be larger than that of HCO_3^- import (Fig. 6*B*). Steady-state pH_i in CO_2/HCO_3^- was slightly higher in *slc4a7*$^{-/-}$ compared with *slc4a7*$^{+/+}$ enterocytes within intact villi (Fig. 6*C*).

We then measured the pH_i recovery rate after an ammonium prepulsing in the presence of both CO_2/HCO_3^- and of inhibitors for the Na^+/H^+ exchanger isoforms NHE1–3. The acid-activated, Na^+-dependent base uptake rate was reduced to almost 50% in *slc4a7*$^{-/-}$-deficient duodenal enterocytes compared with that in *slc4a7*$^{+/+}$ enterocytes, demonstrating that NBCn1 is a major base uptake mechanism in villous duodenocytes in the presence of CO_2/HCO_3^- (Fig. 6*D*).

To assess the importance of NBCn1 for duodenal villus enterocytes in relation to that of other duodenal base uptake mechanisms, we compared the effect of NBCn1 ablation (Fig. 6*D*) with that of NHE1 inhibition (with 1 μM Hoe 642), NHE2 (in addition to NHE1) inhibition (with 50 μM Hoe 642), NHE3 inhibition (by 20 μM S1611), and NBCe1/e2 inhibition (with 200 μM H_2DIDS), in WT mice (Fig. 6*E* and *F*). The results demonstrated that NHE3 and NHE1 are the second most active pH_i recovery mechanisms in duodenal villi, with no contribution of base loaders that are sensitive to low concentrations of H_2DIDS (200 μM), presumably NBCe1 (Praetorius *et al.* 2001) and maybe to some extent the H_2DIDS-sensitive NBCe2 (Virkki

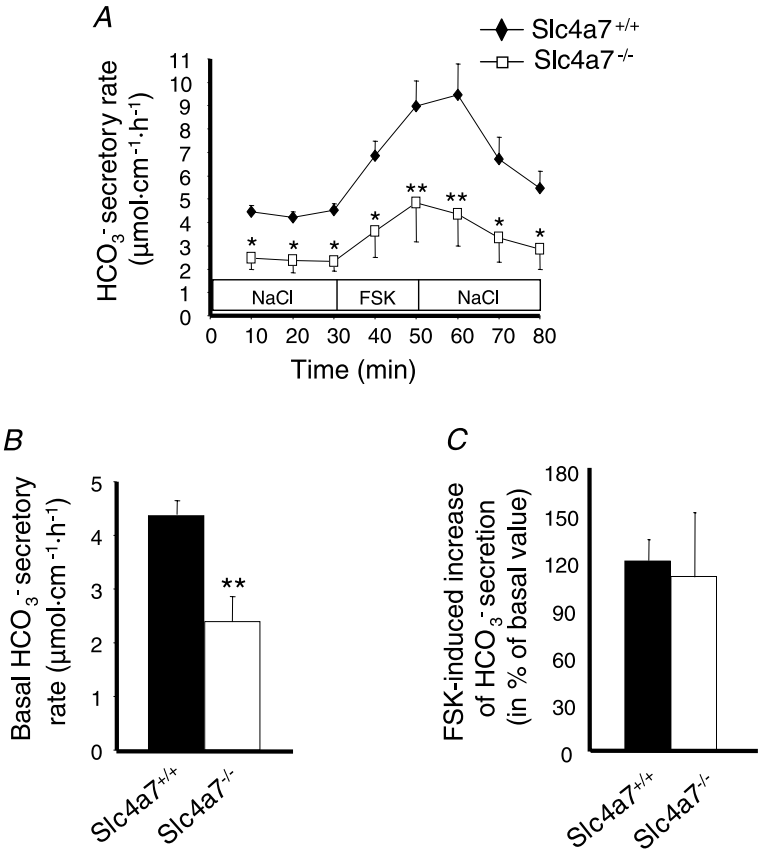

Figure 3. Basal and FSK-activated HCO_3^- secretion in the duodenum of anaesthetized *slc4a7*$^{-/-}$ and *slc4a7*$^{+/+}$ mice
A, time course of duodenal HCO_3^- secretion in anaesthetized *slc4a7*$^{-/-}$ mice and *slc4a7*$^{+/+}$ littermates displayed significantly lower $J_{HCO_3^-}$ at all time points. There was a significant reduction of both basal (Fig. 3*B*) and FSK-stimulated HCO_3^- secretion in the *slc4a7*$^{-/-}$ duodenum, but the HCO_3^- secretory response in per cent above basal (*C*) was not significantly different ($n = 7$ for *slc4a7*$^{-/-}$ and 11 for *slc4a7*$^{+/+}$ mice). $^*P < 0.05$ and $^{**}P < 0.01$.

et al. 2002) in *slc4a7*$^{+/+}$ duodenocytes. Interestingly, there was a significant contribution of a H_2DIDS-sensitive base loader to Na^+ and HCO_3^--dependent pH_i recovery from an acid load in *slc4a7*$^{-/-}$ duodenocytes (Fig. 6*F*), suggesting functional upregulation of NBCe1/e2 under these circumstances. The results demonstrate that NBCn1 is a major, but not the only base uptake mechanism, in villous duodenocytes.

Effect of CA inhibition on basal and FSK-stimulated HCO_3^- secretory rate in the duodenum of *slc4a7*$^{-/-}$ and *slc4a7* $^{+/+}$ mice

As FSK enhanced HCO_3^- secretion even in the *slc4a7*$^{-/-}$ duodeni, alternative HCO_3^- supply mechanisms must exist for the duodenocyte during FSK-stimulated secretion. The potential alternative mechanisms are HCO_3^- uptake by another NBC isoform or CA-facilitated CO_2 hydration and basolateral proton export by Na^+/H^+ exchangers (Jacob *et al.* 2000). In order to distinguish between these possibilities, we repeated the experiments in the presence of luminal and systemic CA inhibitors (which will result in virtually complete CA inhibition; Ilies *et al.* 2000) (Fig. 7). This manoeuvre did not significantly decrease basal or FSK-stimulated HCO_3^- secretion in the WT duodenum (compare the WT values in Fig. 7*A* and *B* with those in Fig. 3*A* and *B*), but significantly reduced FSK-stimulated HCO_3^- secretion in the *slc4a7*$^{-/-}$ duodenum by approximately 50% (compare Fig. 7*C* with Fig. 3*C*). This indicates that CO_2 hydration has no rate-limiting effect in FSK-stimulated duodenal HCO_3^- secretion *in vivo*, but becomes an important pathway for HCO_3^- supply when NBCn1 is not active.

No significant inhibition of basal FSK-induced HCO_3^- secretory rate in isolated slc4a7$^{-/-}$ or slc4a7$^{+/+}$ duodenal mucosa by moderate concentrations of DIDS

As the residual HCO_3^- secretory response suggested the existence of alternative pathways for basolateral HCO_3^- uptake, we studied the effect of 200 μM H_2DIDS in the serosal perfusate (Fig. 8). This concentration of H_2DIDS supposedly inhibits NBCe1 (and NBCe2), while NBCn1

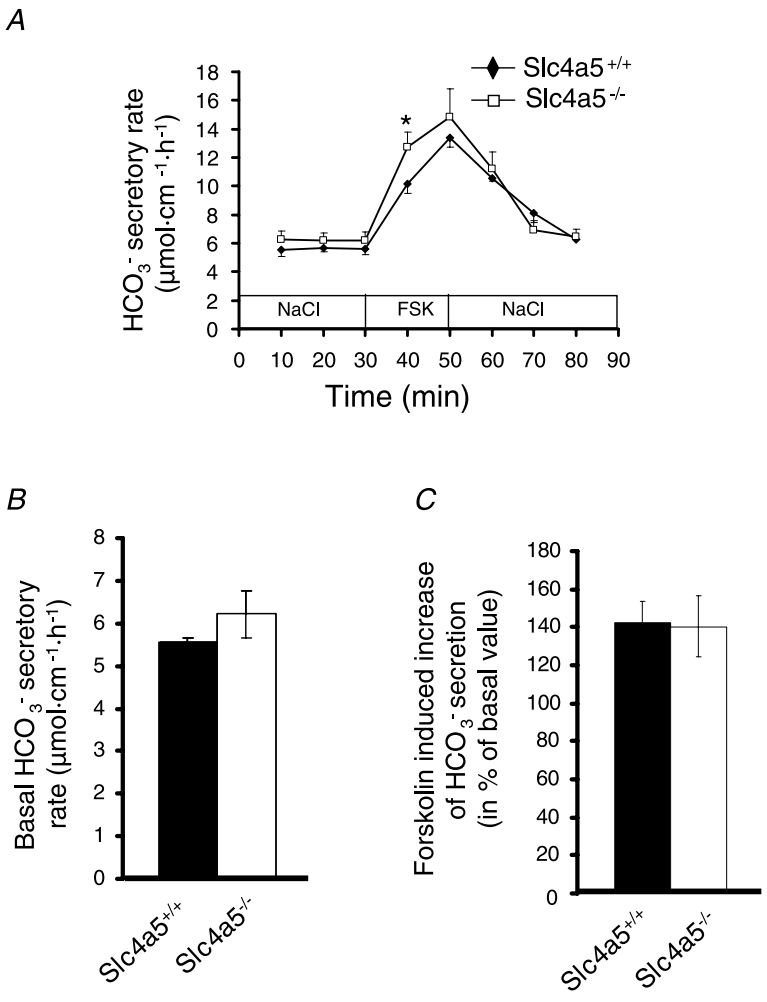

Figure 4. Basal and FSK-activated HCO_3^- secretion in the duodenum of anaesthetized *slc4a5*$^{-/-}$ and *slc4a5*$^{+/+}$ mice
A, time course of duodenal HCO_3^- secretion in anaesthetized *slc4a5*$^{-/-}$ mice and *slc4a5*$^{+/+}$ littermates displayed no difference in $J_{HCO_3^-}$ at any time point. *B*, basal $J_{HCO_3^-}$ and *C*, FSK-stimulated $\Delta J_{HCO_3^-}$ in % of basal were identical in *slc4a5*$^{-/-}$ and *slc4a5*$^{+/+}$ duodenum ($n = 7$ for *slc4a7*$^{-/-}$ and 11 for *slc4a7*$^{+/+}$ mice). The NBCe2 (Slc4a5) gene-targeted mouse model was prepared by deleting the 272-nucleotide exon 13 of Ensemble transcript variant Slc4a5-201 (GenBank Accession number NM_001166067.1). This removed codons 474–563 (beginning with the amino acid sequence ANGLDY and ending with CVAPDT) and created a frameshift that eliminated the potential for translation of the ~500 amino acid membrane-spanning domains of the protein, which are essential for anion transport activity. Details of the gene-targeting strategy and genotyping protocol (E. M. Bradford, Yinhuai Chen and G. E. Shull, unpublished data) will be described elsewhere. The slightly higher $J_{HCO_3^-}$ in these experiments compared with those in Fig. 3 is probably due to the different mouse line, a different investigator, a more advanced anaesthesia equipment and more frequent blood gas analyses.

is relatively insensitive (Burnham *et al.* 1997; Choi *et al.* 2000). H_2DIDS (200 μM) had a dual effect on the I_{sc} response: the addition of H_2DIDS caused a small increase in I_{sc} negativity, and subsequent FSK resulted in a rapid increase to peak values which were not significantly different from the peak values in the respective genotype in the absence of H_2DIDS (compare Fig. 8*C* with Fig. 5*C*), but no sustained plateau phase was observed (as seen in Fig. 5*C*). H_2DIDS, on the other hand, did not significantly inhibit basal or FSK-stimulated HCO_3^- secretory rates (Fig. 8*A* and *B)* in WT and *slc4a7*$^{-/-}$ mucosa when compared with the HCO_3^- secretory responses shown in Fig. 5. This indicates that DIDS-sensitive base importers may not play a significant role as HCO_3^- uptake pathways for FSK-stimulated HCO_3^- secretion in isolated duodenal mucosa. While the addition of 200 μM H_2DIDS did not significantly change the time course of tissue resistance (R_t) when compared with that in the absence of H_2DIDS (compare Fig. 5*D* with Fig. 8*D*), higher concentrations of H_2DIDS resulted in a decrease in R_t, in particular after FSK addition, which limits interpretation (data not shown).

Discussion

The duodenal epithelium is exposed to high concentrations of gastric acid under physiological conditions. Unlike gastric epithelial cells in the fundus region (Wallace, 1989; Boron *et al.* 1994), the duodenal epithelial lining does not possess intrinsic plasma membrane acid resistance (Nylander *et al.* 1994; Akiba *et al.* 2001; Slöblom *et al.* 2009). Consequently, the duodenum needs 'extracellular' as well as 'intracellular' protective mechanisms against acid damage.

It was observed decades ago that disturbances of systemic acid–base balance, with low blood HCO_3^- concentrations, severely endanger the integrity of the duodenal mucosa during gastric acid exposure (Kivilaakso *et al.* 1981). Both a higher epithelial HCO_3^- secretory rate as well as interstitial and intracellular HCO_3^- ions were protective factors (see Allen & Flemstrom, 2005; Kaunitz & Akiba, 2006; Seidler & Sjöblom 2012, for review). This has spurred a strong interest in HCO_3^- secretion as well as HCO_3^- uptake mechanisms of duodenocytes (see Allen & Flemstrom, 2005; Kaunitz & Akiba, 2006; Seidler *et al.*

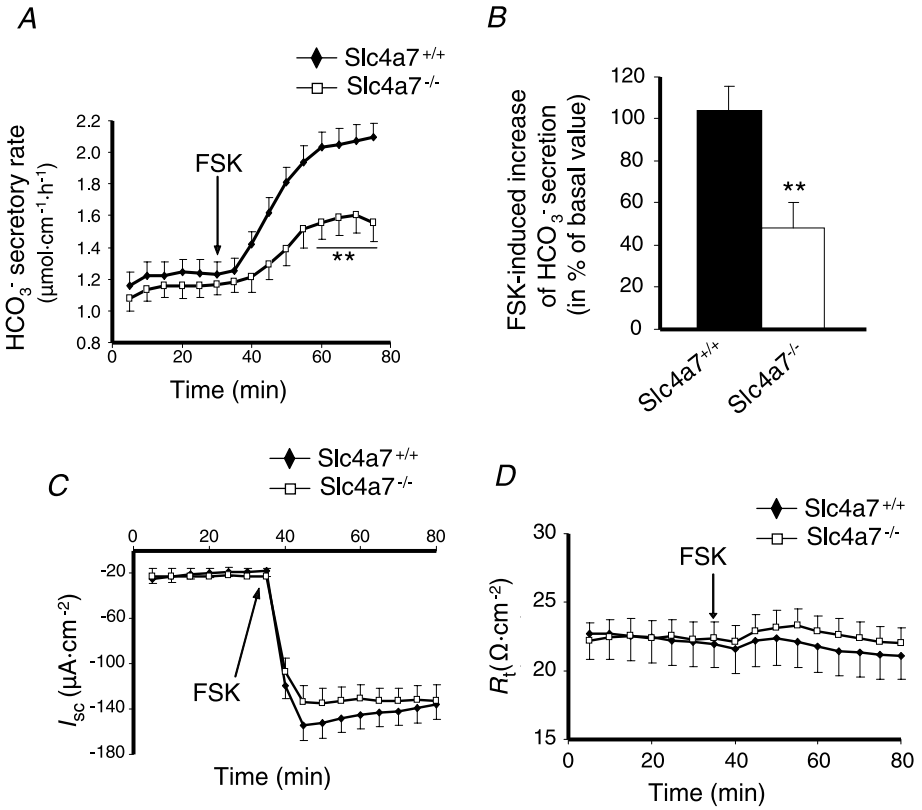

Figure 5. Basal and FSK-stimulated HCO_3^- secretory rates in the isolated duodenal mucosa from *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ mice
Time course of HCO_3^- secretion before and after stimulation with FSK (10^{-5} M in serosal and luminal bath) in isolated *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ duodenal mucosa (*A*). A significantly reduced HCO_3^- secretory response after FSK application was seen in the *slc4a7*$^{-/-}$ duodenum (*B*). The FSK-induced I_{sc} response (*C*), as well as R_t (*D*), was not significantly different in *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ mice ($n = 8$).

2011; Seidler & Sjöblom, 2012, for review). The HCO_3^- supply to the duodenocyte was first thought to depend on CA-mediated CO_2 hydration (Muallem *et al.* 1994; Knutson *et al.* 1995). However, recent work from this laboratory revealed that CA-augmented intracellular CO_2 hydration was essential for 'sensing' the luminal acidity and for responding to it by augmented HCO_3^- secretion, but was not necessary for the HCO_3^- secretory response *per se* (Sjöblom *et al.* 2009).

The involvement of a Na^+:HCO_3^- cotransport system located in the basolateral membrane of the duodenocyte was first discussed in the 1980s during studies of

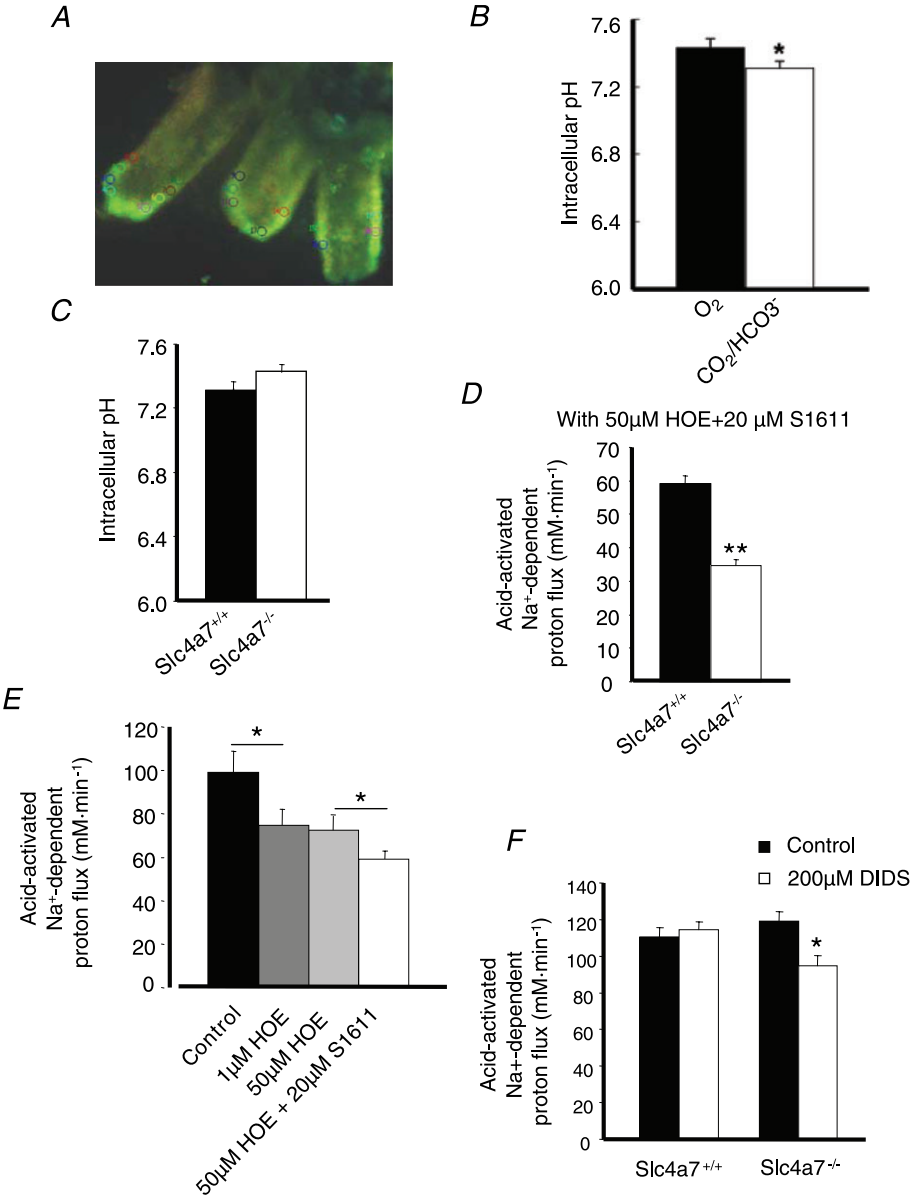

Figure 6. Steady-state pH_i and Na^+-dependent pH_i recovery in enterocytes of intact villus tips
A, BCECF-loaded microdissected duodenal villi. *B*, steady-state pH_i in C57B/6 duodenocytes in the absence and presence of CO_2/HCO_3^-. *C*, steady-state pH_i in villous duodenocytes of *slc4a7*$^{-/-}$ mice in CO_2/HCO_3^--containing buffer was slightly higher than that of *slc4a7*$^{+/+}$ mice. *D*, acid-activated base uptake rates were markedly lower in *slc4a7*$^{-/-}$ compared with *slc4a7*$^{+/+}$ duodenocytes after inhibition of NHE1–3 (by 50 μM of Hoe 642 and 20 μM S1611) in a HCO_3^--containing buffer. *E*, acid-activated, Na^+-dependent base uptake rates in *slc4a7*$^{+/+}$ enterocytes in CO_2/HCO_3^- buffer with sequential inhibition of NHE1, NHE2 and NHE3 (by 1 μM Hoe 642 for NHE1, 50 μM Hoe 642 for NHE1 and NHE2, and 20 μM S1611 for NHE3. *F*, the effect of DIDS on acid-activated base uptake in *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ villi was studied to assess whether the absence of NBCn1 leads to activation of other NBCs. Indeed, 200 mM DIDS caused a significant inhibition of Na^+-dependent base uptake rates in *slc4a7*$^{-/-}$ but not in WT duodenocytes ($n = 3$–5).

isolated amphibian duodenum in Ussing chambers, but the idea was rejected at the time, as experimental evidence for a firm coupling of Na^+ to HCO_3^- transport and SITS sensitivity could not be generated (Simson *et al.* 1981*a*,*b*,*c*). Later, the use of stilbene derivatives and the removal/readdition of CO_2/HCO_3^- to isolated duodenocyte basolateral membranes, duodenal cell suspensions and isolated duodenal mucosa provided functional evidence for the likely involvement of Na^+/H^+ exchangers as well as Na^+:HCO_3^- cotransporters both in duodenocyte pH_i control (Isenberg *et al.* 1993; Ainsworth *et al.* 1998; Praetorius *et al.* 2000, 2001) and for base uptake during HCO_3^- secretion into the lumen (Goddard *et al.* 1998; Jacob *et al.* 2000).

As pointed out in the Introduction, the molecular nature of the $Na^+HCO_3^-$ cotransporter(s) that play a role in duodenal pH_i control and HCO_3^- uptake destined for secretion is under debate. Based on expression studies, NBCe1 (Slca4) and NBCn1 (Slc4a7) are two equally interesting candidates (Fig. 1). NBCe2 (Slc4a5), also reported with intestinal expression (Pushkin *et al.* 2000), was found with lower expression levels (Fig. 1).

Since no pharmacological inhibitors exist for NBCn1, an NBCn1-deficient mouse was created by the insertion of a gene trap into the putative NBCn1 promoter sequence, which resulted in a complete loss of expression of this protein in the duodenum. In the first set of experiments, we searched for a potential role of NBCn1 as a HCO_3^- supply pathway for duodenal HCO_3^- secretion. We found that basal as well as FSK-induced, duodenal HCO_3^- secretion in anaesthetized *slc4a7*$^{-/-}$ mice was significantly reduced compared with WT mice (Fig. 3). This was not explained by changes in systemic acid–base parameters (Table 1). NBCe2 deletion, on the other hand, did not result in significant changes in basal or FSK-stimulated duodenal HCO_3^- secretion (Fig. 4), and since the NBCe2 expression within the murein GI tract was highest in the upper small intestine, yet low compared with NBCe1 and NBCn1 (Fig. 1*A*), the study of this mouse genotype was not pursued further. The Cl^--dependent isoforms Slc4a8 and Slc4a19 had previously been ruled out as major candidates because of low duodenal expression (Damkier *et al.* 2007), the lack of Cl^- dependence of duodenocyte pH_i recovery from an acid load (Ainsworth *et al.* 1998), and because the bilateral removal of Cl^- from the serosal as well as

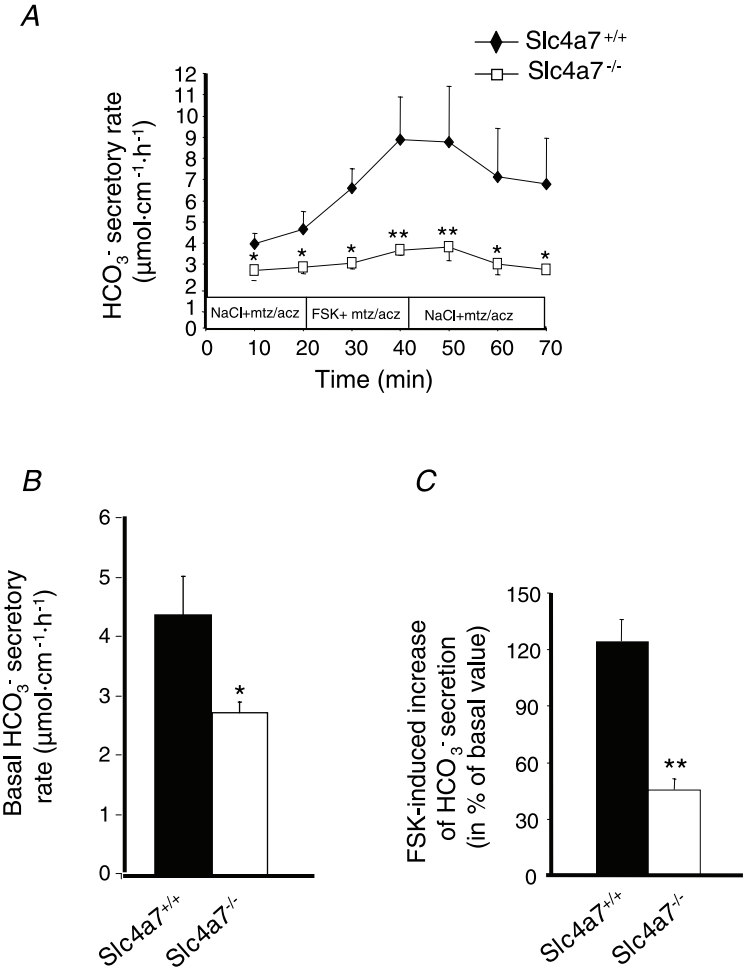

Figure 7. Effect of CA inhibition on basal and FSK-stimulated HCO_3^- secretory rate in the duodenum of *slc4a7*$^{-/-}$ and *slc4a7*$^{+/+}$ mice
A, systemic (10 mg kg^{-1} I.A. acetazolamide) and luminal (1 mM methazolamide) application of CA inhibitors did not significantly inhibit basal or FSK-stimulated duodenal HCO_3^- secretion in *slc4a7*$^{+/+}$ mice, but caused a significant reduction of the duodenal HCO_3^- secretory response to FSK in the *slc4a7*$^{-/-}$ mice. *B*, peak duodenal HCO_3^- secretory rate in *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ mice in % above basal. *C*, the % increase in HCO_3^- secretory rate after FSK was significantly decreased in *slc4a7*$^{-/-}$ compared with *slc4a7*$^{+/+}$ mice ($n = 4$).

the luminal bath did not reduce FSK-induced $\Delta J_{HCO_3^-}$ in rabbit duodenum (Spiegel *et al.* 2003).

The relationship between vascular tone, blood flow and duodenal HCO_3^- secretion is probably highly complex (Jönson *et al.* 1990, 1991). Since NBCn1 is expressed in the vascular system and has recently been shown to play a role in agonist-mediated vasodilatation (Broedtkjer *et al.* 2011), we next assessed whether the effect of *slc4a7* ablation or duodenal HCO_3^- secretory rate was a direct effect due to the lack of this transporter in the isolated epithelium. In the isolated, muscle-stripped and chemically denervated duodenal epithelium, we found a strongly significant reduction in the FSK-elicited HCO_3^- secretory response compared with the WT mucosa (Fig. 5). Thus, NBCn1 is crucial for maximal HCO_3^- secretory rates in the duodenocytes themselves.

To study the role of NBCn1 in duodenocyte pH_i control, fluorometric pH_i measurements were performed in optically isolated duodenocytes in manually dissected intact villi. Steady-state pH_i in WT villous duodenocytes was found to be lower in the presence than in the absence of CO_2/HCO_3^- (Fig. 6*B*) and the absence of NBCn1 did not result in a lower steady-state pH_i (Fig. 6*C*). This indicates that at an external pH of 7.4, the overall action of all HCO_3^- transporters is that of lowering pH_i. This can be due both to electrogenic export of HCO_3^- via CFTR (Simpson *et al.* 2005) and/or NBCe1 (Praetorius *et al.* 2001), or the electroneutral export via Cl^-/HCO_3^- exchange, or all of them. However, NBCn1 was found to be a major pH_i regulator in villous duodenocytes after an intracellular acid load (Fig. 6*D*).

A series of experiments was performed to delineate the other Na^+-dependent base import mechanisms that are active in duodenal villous enterocytes in the presence of CO_2/HCO_3^-. Interestingly, the second most active base uptake mechanisms under the experimental conditions

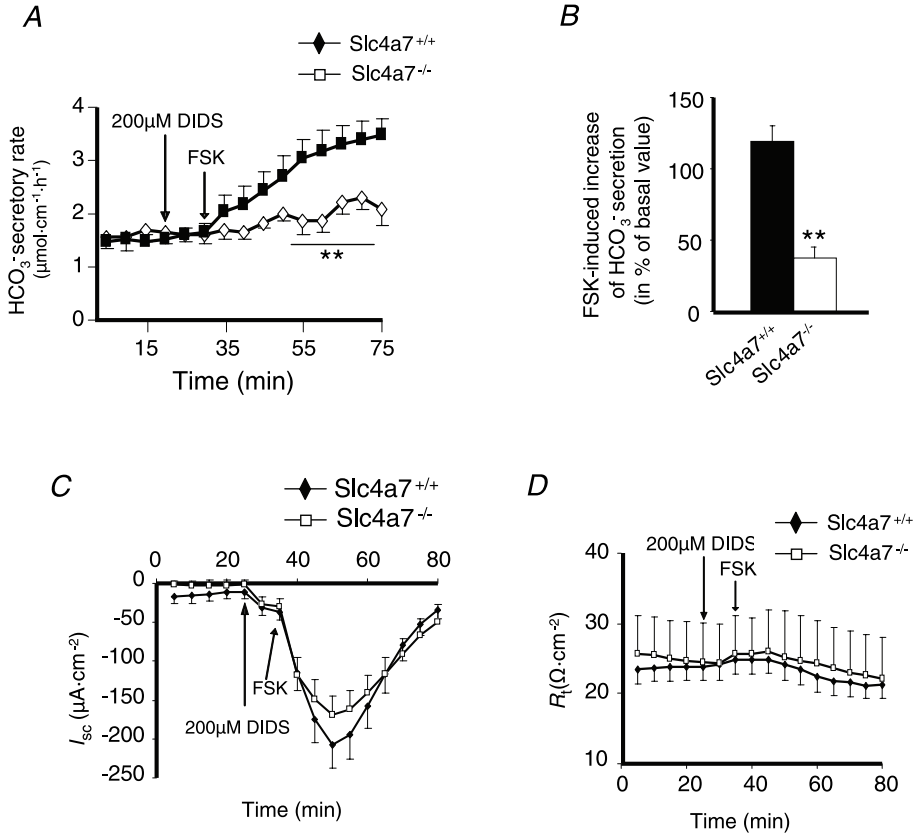

Figure 8. Basal and FSK-stimulated HCO_3^- secretory rates in the isolated duodenal mucosa of *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ mice in the presence of 200 μM H_2DIDS

A, in order to study the contribution of DIDS-sensitive HCO_3^- transporters to the HCO_3^- secretory response, 200 μM H_2DIDS was added to the serosal bath 5 min prior to FSK stimulation, and the per cent of stimulation was calculated for *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ duodenum. *B*, when compared with the results in Fig. 4, 200 μM DIDS did not cause a significant reduction in the FSK-induced HCO_3^- secretory response in *slc4a7*$^{+/+}$ and *slc4a7* $^{-/-}$ duodenal mucosa. However, there was a strong effect of DIDS on the I_{sc} (*C*): I_{sc} became slightly more negative after DIDS application, and the subsequent I_{sc} response to FSK became stronger but declined rapidly, as compared with the stable increase in Fig. 5. R_t was not significantly different in DIDS-treated *slc4a7*$^{+/+}$ and *slc4a7*$^{-/-}$ mice (*D*). $n = 9$

were NHE1 and NHE3 (Fig. 6*E*). This is, to our knowledge, the first investigation of the relative contribution of the different NHE isoforms expressed in the duodenocytes in comparison with that of NBC in the presence of CO_2/HCO_3^-. The potential contribution of the NBCe1 and NBCe2 was assessed by exploiting their sensitivity to low concentrations of the stilbene DIDS (Boyarsky *et al.* 1988; Abuladze *et al.* 1998; Choi *et al.* 1999; Virkki *et al.* 2002). No contribution of DIDS-sensitive NBCs to pH_i recovery from an acid load was observed in WT duodenocytes, despite the obvious cellular and membrane co-expression of NBCe1 with NBCn1 (Figs 1 and 2). Since the membrane potential has been reported to become more negative during an ammonium prepulse as soon as the extracellular NH_4^+ is removed in a variety of cell types (Wu & Vaughan-Jones, 1994; Bonnet & Wiemann, 1999), the electrogenicity of NBCe1- and NBCe2-mediated HCO_3^- transport may make them relatively uneconomical and/or slow for cellular base uptake in comparison with NBCn1. This concept is consistent with the finding of Praetorius *et al.* (2001) of a strong increase in pH_i in isolated duodenocytes upon addition of DIDS, which was discussed at the time as a possible indication of HCO_3^- exit via DIDS-sensitive transporters including NBCes. In the absence of NBCn1, however, the DIDS-sensitive base uptake mechanisms did play a significant role in recovery from acid pH_i (Fig. 6*F*).

As FSK was still able to elicit HCO_3^- secretion in *slc4a7*$^{-/-}$ duodenal mucosa, additional HCO_3^- uptake mechanisms are operative during FSK-stimulated HCO_3^- secretion. The apically located NHE3, which was found to be a major pH_i regulator after intracellular acidification in duodenocytes, is ruled out as a base loader for HCO_3^- secretion because its action has been shown to acidify the lumen (Clarke *et al.* 2001; Furukawa *et al.* 2004; Singh *et al.* 2010). Alternative options are CO_2 diffusion into the enterocyte, intracellular carbonic anhydrase (CA)-facilitated CO_2 hydration, and export of the generated protons via a basolateral NHE (i.e. NHE1), and electrogenic $Na^+(HCO_3^-)_n$ cotransport via NBCe1/NBCe2 (Jacob *et al.* 2000). As the application of systemic DIDS to an anaesthetized mouse will cause multiple confounding effects, we studied the first option *in vivo* and the second one *in vitro*. Duodenal CA inhibition was achieved by luminal and systemic application of non-selective CA inhibitors to *slc4a7*$^{-/-}$ and *slc4a7*$^{+/+}$ anaesthetized mice prior to stimulation of HCO_3^- secretion (Fig. 7). CA inhibition did not significantly affect basal or FSK-stimulated HCO_3^- secretory rates in WT mice, but reduced both parameters by approximately 50% in *slc4a7*$^{-/-}$ mice, indicating that this HCO_3^- supply pathway becomes important in the absence of the major duodenal HCO_3^- importer, NBCn1. This agrees with previously published data from rabbit duodenum on the reciprocal substitution of NBC and NHE/CA for the provision of HCO_3^- during stimulated HCO_3^- secretion (Jacob *et al.* 2000) and suggests that when NBCn1 activity is absent, CA-generated HCO_3^- with H^+ extrusion via NHE1 compensates partially for the HCO_3^- uptake defect (Fig. 9).

The potential contribution of NBCe1/e2 for HCO_3^- supply during FSK-activated HCO_3^- secretion was tested by using the reported DIDS sensitivity of the electrogenic NBCs. However, 200 μM DIDS in the serosal perfusate did not significantly inhibit FSK-stimulated HCO_3^- secretion in WT or in NBCn1-deficient mucosa, and 500 μM DIDS caused an increase in epithelial permeability, but still did not inhibit the FSK-induced HCO_3^- response (data not shown). Therefore, we could not find evidence for a role of NBCe1/e2 for HCO_3^- secretion under the conditions of our experiments. Given the fact that the true sensitivity of the murine pancreatic/intestinal NBCe1 is not known, and DIDS targets many anion transporters but also other proteins, this conclusion is considered provisional and studies in NBCe1-deficient epithelia, possibly generated as intestinal epithelium-specific knockout, are necessary for a final assessment. However, the electrogenicity, the high resting state pH_i in duodenal enterocytes, and the fact that FSK activates basolateral K^+ channels and thus decreases the electrochemical driving force for NBCe1-mediated HCO_3^- uptake, may mask a role for electrogenic NBC in FSK-stimulated duodenal HCO_3^- secretion (Fig. 9). Obviously, this may be different for other NBCe1/e2 expressing epithelia like pancreatic ducts or colonic mucosa.

Why do we see a marked difference in basal HCO_3^- secretory rates between NBCn1 WT and KO mice *in vivo* but not *in vitro*? We know that the 'basal' i.e. non-experimentally stimulated HCO_3^- secretion, *in vivo* is under a 'secretory tone' because it can be inhibited by a variety of manoeuvres, i.e. the application of β-adrenergic blockers (Singh *et al.* 2009) or cyclooxygenase inhibitors (Flemstrom *et al.* 1982). In the experiments in isolated duodenal mucosa, endogenous prostaglandin production was suppressed by indomethacin and any stimulation by neurotransmission blocked by chemical denervation. Thus, the spontaneous secretory tone is presumed to be very low, and indeed basal HCO_3^- secretion of isolated duodenal mucosa is only about one fifth to one tenth of that seen *in vivo*, when calculated in cm^2. Since NBCn1 is not needed to maintain pH_i in ambient CO_2/HCO_3^- and pH (Fig. 6*C*), we assume that this transporter becomes important when cellular HCO_3^- is lost, either by export into the lumen or during cellular acidification. Thus, its importance is seen *in vivo* without additional external stimulation, but in isolated duodenal mucosa only when HCO_3^- secretion is experimentally stimulated.

The conceptual differences between measuring duodenal HCO_3^- secretion *in vivo* and in the isolated mucosa also need to be discussed. The isolated

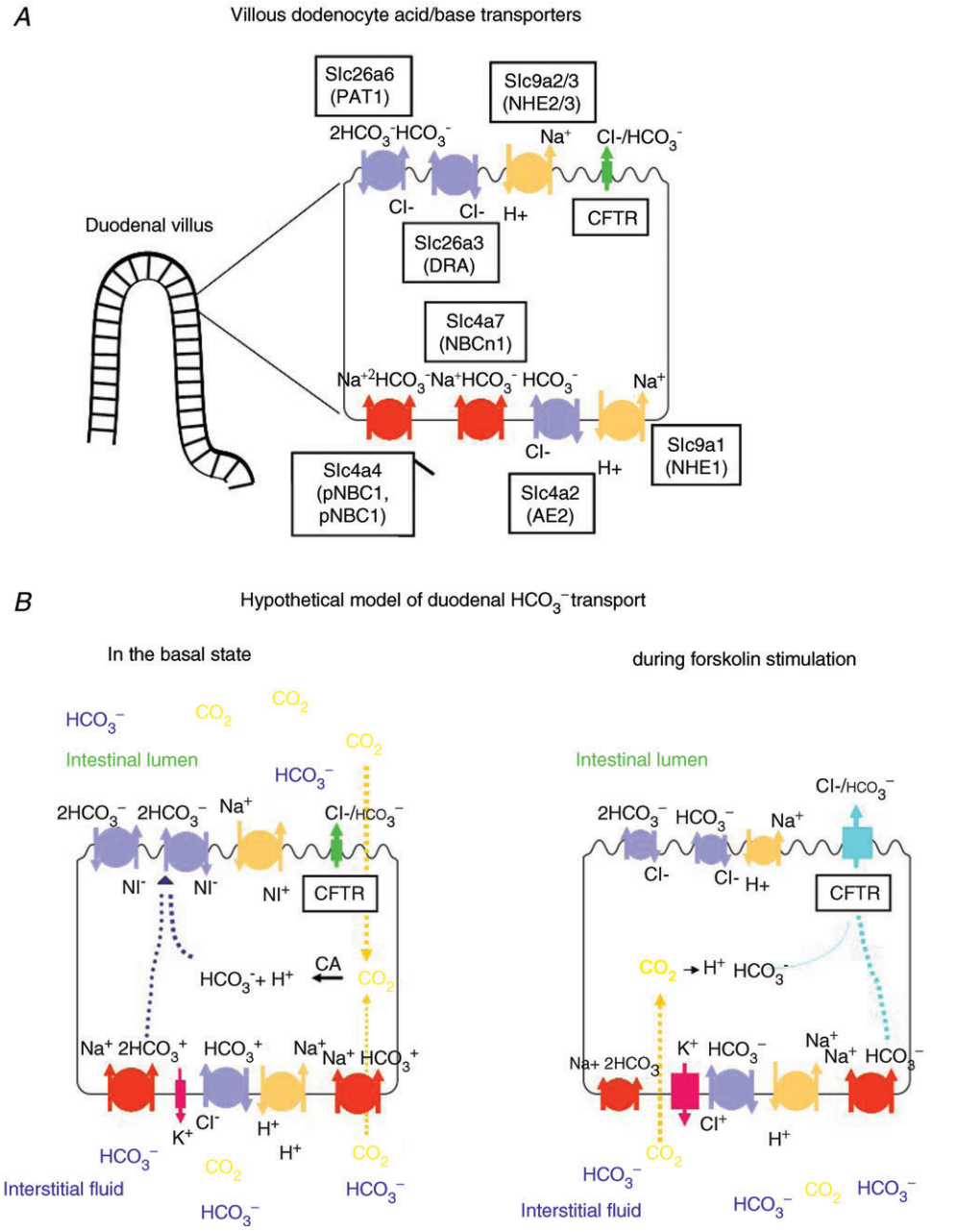

Figure 9. Model of the localization and function of villous duodenocyte acid–base transporters
A, apical and basolateral ion transporters that result in movement of HCO_3^- across the membrane, either directly or via proton movement, with demonstrated localization in villous duodenocytes (Jacob *et al.* 2000, 2002; Praetorius *et al.* 2000; Wang *et al.* 2002; Simpson *et al.* 2005; Walker *et al.* 2008; Jakab *et al.* 2011; this paper). *B*, hypothetical model depicting the major route(s) of bicarbonate entry into and exit from the villous duodenocyte in the resting state (left panel) and during FSK stimulation (right panel). Resting state is defined as: nutrient-free interdigestive phase, no acid discharge from the stomach, no massive HCO_3^- output from the pancreas, no neurohumoral stimulation of epithelial secretion. The villous duodenocyte is in an absorptive state. The major HCO_3^- exit pathway is probably Slc26a3 (DRA), with minor HCO_3^- exit via CFTR. HCO_3^- will be generated from CO_2 hydration, with the CO_2 being recycled from the lumen, as well as diffusing from the vasculature/interstitium. HCO_3^- will also enter the cell, possibly via NBCe1 > NBCn1 (Na^+ as well as Cl^- are absorbed from the lumen, and a conductive basolateral Cl^- exit may lower membrane potential, only constitutive basolateral K^+ channels active). After FSK stimulation, apical CFTR is phosphoryated and may become more HCO_3^- permeable secondary to WNK-SPAK/OSR1 activation (Park *et al.* 2010). The large HCO_3^- efflux via CFTR and the FSK-mediated inhibition of NHE3 will both inhibit HCO_3^- efflux via Slc26a3, but stimulate HCO_3^- uptake via NBCn1 >NBCe1 (this paper). FSK also activates basolateral K^+ channels, which may result in less favourable conditions for NBCe1- than NBCn1-mediated HCO_3^- uptake.

small intestinal murine mucosa in the Ussing chamber is well oxygenated due to its extreme thinness. The epithelium remains in a net salt absorptive mode for hours, suggesting an intact energy supply and tight junctional integrity (Clarke & Harline, 1996; Seidler *et al.* 2008; Walker *et al.* 2008). However, for certain substances, there may nevertheless be diffusion from the serosal side to the villous tips. While the problem with agonist diffusion in isolated chambered duodenum may be solved by adding the agonist to both compartments, the same problem may exist with HCO_3^-, and this cannot be solved experimentally, since the lumen needs to be unbuffered in order to be able to titrate HCO_3^- secretion. In addition, CO_2 from the lumen has been shown to enhance duodenal HCO_3^- secretion (Furukawa *et al.* 2005), but it also enhances apical Na^+/H^+ exchange and thus HCO_3^- absorption from the lumen. (Hubel, 1973; Turnberg *et al.* 1970; Xia *et al.* Xia, W, Yu, Q, Seidler U unpublished observations). FSK is known to inhibit luminal Na^+/H^+ exchange and may thus mimick a 'HCO_3^- secretory response'. This mechanism is probably completely missing or greatly diminished in the Ussing chamber, because the large reservoir is continuously gassed and any CO_2 diffusion from the serosal bath will be eliminated. It is therefore unclear what the HCO_3^- concentration will be at the basolateral membrane of upper villi duodenocytes under those circumstances. Thus, working with isolated epithelium to study small intestinal HCO_3^- secretion may result in a relative overestimation of what occurs in the crypt/lower villous region and underestimation of the processes in the upper villous region.

Measurements of duodenal bicarbonate secretion *in vivo* also have confounding factors, most prominently due to the complexity of signalling in the whole organism. Firstly, the application of agonists may change HCO_3^- secretory rates not only by changing the ion transport rates at the epithelial level, but also by changing HCO_3^- transport to the epithelium, via changes in vascular and systemic acid–base parameters. Secondly, anaesthesia may compromise the respiratory pattern of the mice, resulting in hyper- or hypoventilation, and this compromises acid–base balance and changes duodenal HCO_3^- secretion. We therefore record the ventilation frequency, adjust the isoflurane concentration accordingly, infuse base if necessary, and take blood gas measurements. In the luminally perfused duodenum *in vivo*, we assume that CO_2 diffusion into the rather narrow lumen is rapid, ambient P_{CO_2} probably close to identical in blood and lumen, and luminal CO_2 available for HCO_3^- supply to the villous enterocytes. We also know that the *in vivo* 'basal' HCO_3^- secretory rate is actually under a 'secretory tone', as shown by further inhibition by ß-adrenergic antagonists (Singh *et al.* 2009). Therefore, what we call 'basal HCO_3^- secretory rate' *in vivo* may actually to some extent be activated, FSK-induced $\Delta J_{HCO_3^-}$; on the other hand, it may in part result from an inhibition of apical Na^+/H^+ exchange. Significant differences in 'basal HCO_3^- secretion' between KO and WT *in vivo* but not *in vitro* may be due to the *in vivo* 'HCO_3^- secretory tone', whereas a significant percental increase in $\Delta J_{HCO_3^-}$ *in vitro but not in vivo* may be due to a larger interference of FSK with apical proton secretion *in vivo* than *in vitro*. To ascertain that NBCn1 is a physiologically important base uptake mechanism during duodenal HCO_3^- secretion, we have therefore measured basal and FSK-induced duodenal HCO_3^- secretion in *slc4a7*$^{-/-}$ mice and WT littermates both *in vivo* and in isolated mucosa *in vitro*. The combination of both methods seems most suitable to fully describe the HCO_3^- secretory and pH_i regulatory defect in *slc4a7*$^{-/-}$ murine duodenum.

In summary, this study demonstrates a prominent role for the villus-expressed electroneutral Na^+:HCO_3^- cotransporter derived from the *Slc4a7* gene as a major pH_i regulatory mechanism in murine villous duodenocytes. Furthermore, NBCn1 is essential to maintain basal and stimulated HCO_3^- secretory rates. Alternative HCO_3^- uptake mechanisms, such as CA-mediated CO_2 hydration or the equally expressed electrogenic NBCe1, are functionally up-regulated in its absence, but cannot maintain normal HCO_3^- secretory rates. However, more insight into the regulation of NBCn1 and NBCe1 under different physiological conditions is needed to fully understand the reason for the equally strong expression of these two HCO_3^- transporters in the villous duodenocyte basolateral membrane.

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