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Molecular transport machinery involved in orchestrating luminal acid-induced duodenal bicarbonate secretion *in vivo*

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Key points

- Acid damage of the proximal duodenum is a key pathogenic factor in duodenal ulcer disease as well as in the intestinal manifestations of cystic fibrosis.
- Short contact of healthy duodenal mucosa with acid results in long-lasting stimulation of proximal duodenal bicarbonate secretion.
- While the complex neural, paracrine, humoral and luminocrine regulation of this acid-induced bicarbonate secretory response, as well as its protective role, has been studied in some detail, little is known about the molecular identity of the involved ion transporters or intracellular signalling.
- Using genetically engineered mouse models, we found that the anion exchanger DRA (Slc26a3), the anion conductances Slc26a9 and cystic fibrosis transmembrane conductance regulator, and the Na^+/H^+ exchanger isoform 3 play essential roles in orchestrating the acid-induced duodenal bicarbonate secretory response. These transporters are differentially controlled by signalling mechanisms along the crypt–villus axis.
- These findings provide a better understanding of the pathophysiology of peptic damage to the duodenum and may provide novel treatment strategies.

Abstract The duodenal villus brush border membrane expresses several ion transporters and/or channels, including the solute carrier 26 anion transporters Slc26a3 (DRA) and Slc26a6 (PAT-1), the Na^+/H^+ exchanger isoform 3 (NHE3), as well as the anion channels cystic fibrosis transmembrane conductance regulator (CFTR) and Slc26a9. Using genetically engineered mouse models lacking Scl26a3, Slc26a6, Slc26a9 or Slc9a3 (NHE3), the study was carried out to assess the role of these transporters in mediating the protective duodenal bicarbonate secretory response (DBS-R) to luminal acid; and to compare it to their role in DBS-R elicited by the adenylyl cyclase agonist forskolin. While basal DBS was reduced in the absence of any of the three Slc26 isoforms, the DBS-R to forskolin was not altered. In contrast, the DBS-R to a 5 min exposure to luminal acid (pH 2.5) was strongly reduced in the absence of Slc26a3 or Slc26a9, but not Slc26a6. CFTR inhibitor [CFTR(Inh)-172] reduced the first phase of the acid-induced DBS-R, while NHE3 inhibition (or knockout) abolished the sustained phase of the DBS-R. Luminal acid exposure resulted in the activation of multiple intracellular signalling pathways, including SPAK, AKT and p38 phosphorylation. It induced a biphasic trafficking of NHE3, first rapidly into the brush border membrane, followed by endocytosis in the later stage. We conclude that the long-lasting

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DBS-R to luminal acid exposure activates multiple duodenocyte signalling pathways and involves changes in trafficking and/or activity of CFTR, Slc26 isoforms Slc26a3 and Slc26a9, and NHE3.

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Abbreviations BBM, brush border membrane; CFTR, cystic fibrosis transmembrane conductance regulator; DBS, duodenal bicarbonate secretion; DBS-R, duodenal bicarbonate secretory response; FSK, forskolin; KO, knockout; NHE, sodium/hydrogen exchanger; OSR, oxidative stress-responsive kinase; PGE_2, 16,16-dimethyl prostaglandin E_2; PKA, protein kinase A; Slc, solute carrier; SPAK, STE20/SPS1-related proline/alanine-rich kinase; WNK, with no lysine kinase; WT, wild-type

Introduction

Duodenal mucosal bicarbonate (HCO_3^-) secretion (DBS) is considered to be one of the most important defence mechanisms against gastric acid by maintaining an alkaline microclimate near the apical epithelial membrane (Allen & Flemstrom, 2005; Kaunitz & Akiba, 2006; Seidler & Sjöblom, 2012). Different agonists were found to differentially regulate the electrogenic (presumably anion channel dependent) and electroneutral (presumably Cl^-/HCO_3^- exchanger-dependent) HCO_3^- transport pathways (Allen & Flemström, 2005; Seidler & Sjöblom, 2012).

Various members of the Slc26 family of anion transporters, the Cl^-/HCO_3^- exchangers Slc26a6 (PAT1) (Tuo *et al.* 2006) and Slc26a3 (DRA) (Jacob *et al.* 2002; Walker *et al.* 2009), are involved in basal DBS. Another member of the same family, Slc26a9, whose transport properties are more diverse (Xu *et al.* 2005; Dorwart *et al.* 2007; Bertrand *et al.* 2009; Chang *et al.* 2009), is expressed at low level in the duodenum (Xu *et al.* 2005). The physiological stimuli that may activate Slc26a3, Slc26a6 or Slc26a9-mediated HCO_3^- transport have not been identified.

The Na^+/H^+ exchanger isoform 3 (NHE3) is located in the duodenal villus brush border membrane (BBM). The pharmacological inhibition of NHE3 increases basal DBS (Repishti *et al.* 2001; Furukawa *et al.* 2004; Singh *et al.* 2010), at least in part through inhibition of proton extrusion via the exchanger. However, whether NHE3 inhibition is an actively regulated mechanism involved in acid-induced DBS-R remains unknown.

Contact of the duodenal mucosa with acid in the lumen stimulates DBS-R in all species tested, including humans (Allen & Flemström, 2005; Seidler & Sjöblom, 2012). This acid-induced DBS-R involves stimulation of neural circuits (Takeuchi *et al.* 1991; Singh *et al.* 2012), and can therefore only be tested *in vivo*. While multiple stimulatory pathways may activate different receptors along the crypt–villus axis, the ion transport mechanisms are largely unknown. Several studies demonstrated the importance of cystic fibrosis transmembrane conductance regulator (CFTR) anion channel expression in acid-induced DBS-R (Hogan *et al.* 1997; Singh *et al.* 2008). The potential role of other ion transport mechanisms in acid-induced DBS-R has not been elucidated.

The aim of this study was to identify the apical acid/base transport proteins involved in acid-induced DBS-R, using genetic knockout and selective pharmacological inhibition of members of the Slc26 family, of NHE3 as well as CFTR. In an attempt to understand why the short contact of, predominantly, the upper villus epithelium with acid results in complex transport changes of multiple ion transporters with differential distribution along the crypt–villus axis, we also investigated signalling pathway activation in the duodenal epithelium.

Methods

Chemicals and solutions

All the reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany) unless mentioned otherwise. Forskolin (FSK) was purchased from Alexis Biochemicals (Lörrach, Germany). Stock solution of FSK was prepared and stored at −20°C. It was dissolved in 100% dimethyl sulphoxide (10^{-2} M) and used at a final concentration of 100 μM. Stock solution (10^{-3} M) of 16,16-dimethyl prostaglandin E_2 (PGE_2) was prepared in absolute ethanol and stored at −20°C. PGE_2 was used at a final concentration of 1 μM. A stock solution (5 mg ml^{-1}) of indomethacin was prepared in saline and a dose of 5 mg (kg body weight)$^{-1}$ was given by intraperitoneal injection 1 h before the experiment. Primary antibodies were purchased from: CFTR (http://cftrfolding.org, Cat no. 3G11, California, USA), NHE3 (Alpha Diagnostic International, Cat no. NHE31_A, Cologne, Germany), WNK1 (Abnova, Cat no. PAB18141, Heidelberg, Germany), WNK4 (ABBIOTEC, Cat no. 250912, Cologne, Germany), SPAK (Cell Signaling, Cat no. 2281, Frankfurt, Germany), pp38 (Cell Signaling, Cat no. 9215, Frankfurt, Germany), pAKT(Thr308) (Cell Signaling, Cat no. 2965, Frankfurt, Germany), pAKT(Ser473) (Cell Signaling, Cat no. 9271, Frankfurt, Germany), β_actin (abcam, Cat no. ab8227, Cambridge, UK). pSPAK/pOSR1 (kind gift from Min Goo Lee; Park *et al.* 2010). Goat antirabbit Alexa 488 (Invitrogen, catalogue no. A11008, Frankfurt,

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Germany) was used as secondary antibody in the immunohistochemistry and goat antirabbit IgG conjugated to horseradish peroxidase (KPL, catalogue no. 214-1516, Wedel, Germany) used as secondary antibody in Western blot analysis.

Ethical approval

All studies were approved by the Hannover Medical School Committee on investigations involving animals and the Lower Saxony Institute for Verbraucherschutz and Veterinary Medicine. Mice were killed with CO_2 or isoflurane narcosis followed by cervical dislocation. The experimental procedures performed and types of anaesthesia were used according to university and national guidelines and are explained below.

Animals

Experiments were performed with wild-type (WT) and gene-deficient knockout (KO) mouse models of Slc26a3, Slc26a6, Slc26a9, Slc9a3 (NHE3), Slc9a2 (NHE2) and KCNQ1 (Schweinfest *et al.* 1993; Schultheis *et al.* 1998; Casimiro *et al.* 2001; Wang *et al.* 2005; Xu *et al.* 2008). Mice were bred at the animal care facility of Hannover Medical School under standard temperature and light conditions and were allowed free access to food and water. The Slc26a3, Slc26a6, Slc9a3 and KCNQ1 WT and KO mice were congenic on C57BL/6N background, whereas the Slc26a9 were congenic on the SV129J background. Slc9a2 WT and KO mice were congenic on the NMRI background. The Slc26a3 mice (WT and KO) were fed a special diet (Altromin, Lippe, Germany, catalogue no. C0197) and drinking water containing (KCl 10 mM, NaCl 7.5 mM, sodium citrate 5 mM and sucrose 35 mM), which enables survival beyond weaning. The Slc9a3 (WT and KO) were also fed a special diet (Altromin, catalogue no. 1057). Care was taken to ensure an approximately equal number of male and female pairs of WT and KO mice in each group of experiments. For experiments that only used WT mice (with pharmacological inhibitors), the C57BL/6N background was used. All experiments were performed in mice aged between 10 and 20 weeks. We tried to use littermates, if that was not possible we used WT mice of similar age/gender, from the same grand-parental lineage.

Surgical procedure '*in vivo* duodenal loop experiment'

The surgical procedure were performed exactly as has been previously described (Singh *et al.* 2010, 2012).

Measurement of luminal alkalinization

The DBS was determined by back-titration of the perfusate to pH 5.00 with 2 mM HCl using pH-stat equipment (PHM82 STANDARD pH meter Radiometer, Copenhagen, Denmark) as described (Singh *et al.* 2009, 2010, 2012). For PGE_2 stimulation, the mice received a 5 mg (kg body weight)$^{-1}$ intraperitoneal injection of indomethacin 1 h before the experiment to inhibit endogenous PGE_2 production.

Fluid absorption measurement

For duodenal fluid absorption, approximately 3–5 cm of intestine starting from the pylorus was perfused at a rate of 3 ml h^{-1} with 154 mM NaCl, as described in Singh *et al.* (2010).

Measurement of mRNA expression of Slc26a3, Slc26a6, Sl26a9, Slc9a3, CFTR, NBCn1 and NBCe1

mRNA expression levels in scraped duodenal mucosa was performed using a quantitative real-time polymerase chain reaction protocol as previously described (Broere *et al.* 2009). The primer pairs for Slc26a6 (Alvarez *et al.* 2004), villin, Slc9a3 (NHE3) (Broere *et al.* 2009), NBCe1 and NBCn1 (Chen *et al.* 2012) have been published. Primer sequences for other genes are provided in the supplementary file (Supplementary Table 1).

Immunohistochemistry

The protocol for Slc9a3 (Cinar *et al.* 2007) and CFTR (Singh *et al.* 2009) immunohistochemistry has been described before. For SPAK, pSPAK/pOSR1, pp38, pAKT (Thr308), WNK1 and WNK4, the same protocol was used as for Slc9a3 (NHE3). The localization of the NHE3 as compared to F-actin (terminal web) was assessed by ImageJ (NIH, Bethesda, Maryland, USA) software as has been previously described in detail (Chen *et al.* 2010). The peak value of the F-actin profile was taken as reference for plotting the NHE3 profile. For each condition a minimum of 15 cells were taken into consideration from each image. The graph shows the mean of the intensity profiles.

Western analysis

In vivo perfusion was performed as described above. Control mice received luminal perfusion with isotonic saline for 40 min. The experimental groups of mice were perfused with saline for 20 min, followed by either of the solutions: isotonic sodium gluconate (150 mM), hypotonic sodium gluconate (50 mM), saline containing 100 μm FSK and 5 min pH 2.5 saline. Mice were killed immediately after the acid pulse (0 min), and at 10 and 30 min intervals, or 20 (FSK) and 40 (sodium gluconate) min after the solution switch. After killing the perfused segment was excised and the mucosa scraped, homogenized, lysed in 2× sodium dodecyl sulphate buffer [100 mM Tris-Cl (pH 6.8), 4% (w/v) sodium dodecyl sulphate, 0.2% bromophenol blue, 20% (v/v) glycerol, 200 mM DTT

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(which was added fresh)], heated at 95°C for 5 min, centrifuged at 19,000 g for 15 min to remove cellular debris, and stored at −80°C until further use. Western analysis was performed, using pAKT (Thr308), pAKT (Sre473), pSPAK/pOSR1, pp38 and β-actin antibody, as previously described (Singh *et al.* 2009). For the quantification of the band ImageJ software was used.

Statistical analysis

Values are expressed as means ± S.E.M., with the number of experiments (mice or WT and KO pairs) given in parenthesis. Statistical comparisons were either made using a two-tailed, unpaired Student's t test for single value comparisons or by ANOVA with *post hoc* analysis for multiple comparisons. Data were considered significant when $P < 0.05$.

Results

Involvement of Cl^-/HCO_3^- exchanger during basal duodenal bicarbonate secretion and forskolin- and acid-stimulated duodenal bicarbonate secretory response

After removal of Cl^- from the luminal perfusate, basal DBS was reduced to almost zero (Fig. 1*A*), but FSK-induced DBS-R was not different from that in the presence of luminal Cl^- (Fig. 1*A* and *B*). The CFTR inhibitor [CFTR(Inh)-172] strongly inhibited the DBS-R to FSK in the absence of luminal Cl^-, suggesting that under these circumstances, CFTR activity is necessary and may be a major export pathway for the secreted HCO_3^-.

In contrast, acid-stimulated DBS-R was dramatically reduced in the absence of luminal Cl^- (Fig. 2*A* and *B*), indicating a potential involvement of Cl^-/HCO_3^- exchangers in mediating acid-induced DBS-R. CFTR-(inh)-172 also significantly inhibited the acid-stimulated DBS-R (Fig. 2*A* and *B*).

Involvement of Slc26a3, a6 and a9 in the forskolin-stimulated duodenal bicarbonate secretion

In the absence of Slc26a3, basal DBS was reduced to almost 50%, but no significant difference was observed in the FSK-induced DBS-R between Slc26a3-deficient and WT duodenum (Fig. 3*A*). No significant differences were observed in either basal or FSK-induced DBS-R in Slc26a6 duodenum (Fig. 3*B*). A significant reduction in basal DBS was also observed in the Slc26a9-deficient duodenum (Fig. 3*C*), while the FSK-stimulated DBS-R was not different *vs*. WT duodenum.

Involvement of Slc26a3, a6 and a9 in acid-stimulated duodenal bicarbonate secretion

After a 5 min contact of the duodenal mucosa with pH 2.5 saline, Slc26a3-deficient mice showed a significant decrease in acid-stimulated DBS-R (Fig. 4*A*), while Slc26a6-deficient mice did not (Fig. 4*B*). Very little acid-induced DBS-R was observed in the absence of Slc26a9 (Fig. 4*C*).

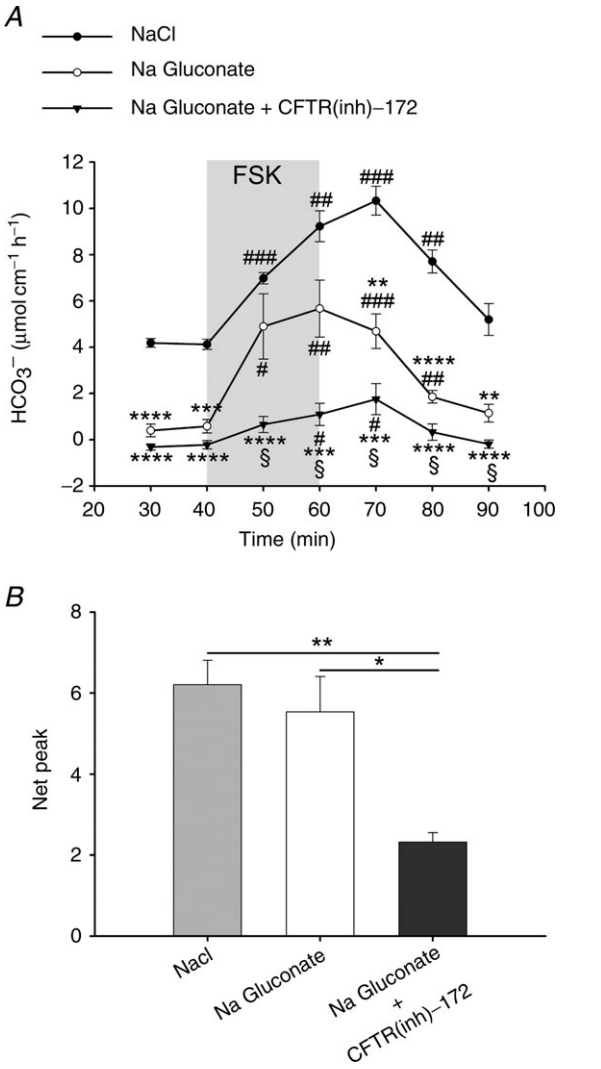

Figure 1. Basal DBS but not FSK-stimulated DBS-R is dependent on luminal Cl^-
A, time course of basal DBS and FSK-stimulated DBS-R (100 μM in luminal perfusate present during the time segment indicated by the shaded area) in C57BL/6N mice. No difference was observed in the magnitude of FSK-induced DBS-R in the absence and presence of luminal Cl^- (*A* and *B*). CFTR inhibitor [CFTR(inh)-172, 40 μM luminally] significantly inhibited FSK-induced DBS-R, suggesting that a functional CFTR is involved. Values are presented as means ± S.E.M. #Significant increase as compared to basal within the same group. *Significant decrease as compared to the mice perfused with saline. §Significant decrease as compared to the mice perfused with sodium gluconate *vs*. CFTR inhibitor. *, #, §$P < 0.05$; ##, **$P < 0.01$; ###, ***$P < 0.001$; ****$P < 0.0001$; $n = 4$–6. CFTR, cystic fibrosis transmembrane conductance regulator; DBS-R, duodenal bicarbonate secretory response; FSK, forskolin.

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Involvement of Slc26a9 in prostaglandin E_2-stimulated duodenal bicarbonate secretion

Endogenous PGE_2 release has been shown to occur during duodenal mucosal acidification (Sugamoto *et al.* 2001), suggesting that low concentrations of PGE_2

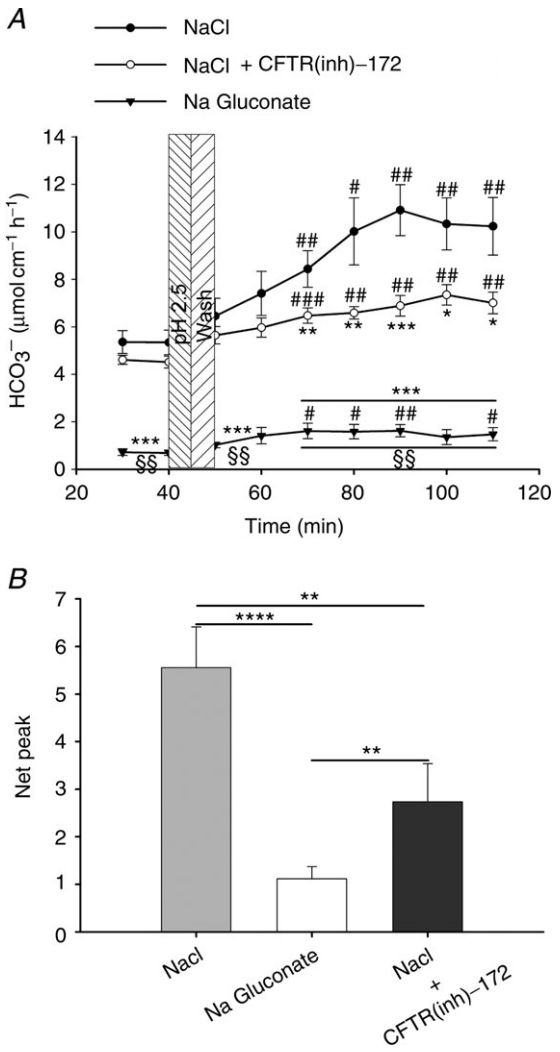

Figure 2. Acid-induced DBS-R displays strong dependency on luminal Cl^-
A, time course of basal DBS and acid-induced DBS-R in C57BL/6N mice. A strong difference in acid-induced DBS-R was observed between the absence and presence of luminal Cl^-, suggesting the involvement of Cl^-/HCO_3^- exchanger. CFTR(inh)-172 significantly inhibited the acid-stimulated response in saline-perfused mice, suggesting the additional involvement of CFTR. *B*, acid-induced DBS-R, given as the peak response minus the mean basal rate for each experiment for the different conditions. Values presented as means ± S.E.M. #Significant increase as compared to basal within the same group. *Significant decrease as compared to the mice perfused with saline. §Significant decrease as compared to the mice perfused saline with CFTR inhibitor *vs.* sodium gluconate. #, $^*P < 0.05$; ##, §§, $^{**}P < 0.01$; ###, §§§, $^{***}P < 0.001$; $n = 5$. CFTR, cystic fibrosis transmembrane conductance regulator; DBS-R, duodenal bicarbonate secretory response.

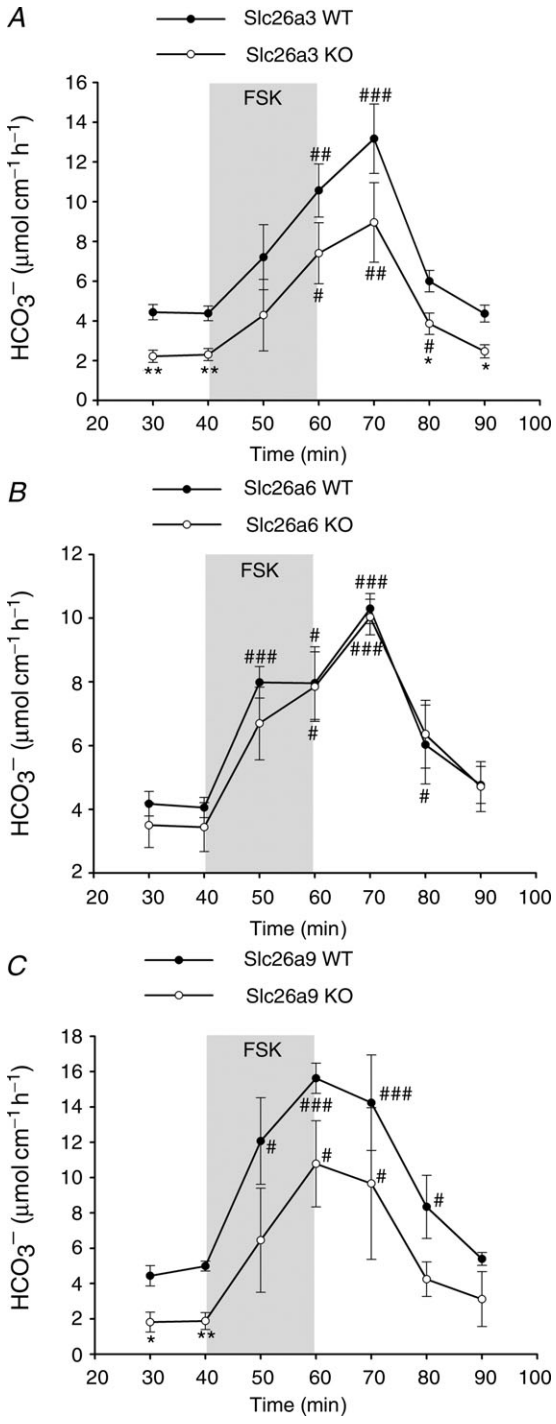

Figure 3. Basal DBS and FSK-stimulated DBS-R in Slc26a3, a6 and a9 KO and WT mice
A, basal DBS and FSK-induced DBS-R in Slc26a3 KO and WT mice. *B*, in Slc26a6 KO and WT mice. *C*, in Slc26a9 KO and WT mice. While basal DBS was significantly lower in Slc26a3 and Slc26a9 KO mice, FSK-stimulated DBS-R was not different from WT in the three different Slc26 KO groups. Values presented as means ± S.E.M.# Significant increase as compared to the basal with in the same group. *Significant decrease in KO mice as compared to the WT littermates. #, $^*P < 0.05$; ##, $^{**}P < 0.01$; ###$P < 0.001$; $n = 4$–6. DBS-R, duodenal bicarbonate secretory response; FSK, forskolin; KO, knockout; WT, wild-type.

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mimic physiological acid stimulation (Hirokawa *et al.* 2004). As acid-stimulated duodenal HCO_3^- secretion in Slc26a9-deficient mice was strongly reduced, whereas FSK-induced HCO_3^- secretion was normal, we tested PGE_2 (1 μM)-stimulated DBS-R in Slc26a9-deficient mice. The DBS-R to luminal application of 1 μM PGE_2 was strongly reduced in the absence of Slc26a9 compared to WT (Fig. 4*D*).

Histological appearance of Slc26a3-, Slc26a6- and Slc26a9-deficient duodenum

Histological assessment of the proximal duodenum of Slc26a3-, Slc26a6- and Slc26a9-deficient duodenum revealed no obvious differences between WT and gene-deficient duodenal mucosae (Supplementary Fig. 1*A*–*D*).

Duodenal fluid transport during forskolin and acid-induced bicarbonate secretory response

Net fluid transport rates in perfused duodenum of anaesthetized mice was studied by single pass perfusion (Fig. 5). In the basal state, the duodenum absorbed fluid. In the first 30 min after acid exposure, only a small reduction in fluid absorptive rate was seen, with a somewhat stronger reduction in the second 30 min observation period. In contrast, a massive fluid secretory response was observed in the first 30 min after FSK application. This suggests that luminal acid exposure weakly stimulates the events that cause fluid secretion, whereas FSK is a strong stimulant.

Pharmacological inhibition or genetic deletion of sodium/hydrogen exchanger isoform 3 alters the extent and time course of acid-induced bicarbonate secretory response

The results described in the previous sections suggested that the acid-induced HCO_3^- secretory response might possibly be of villous origin (involvement of villus-based Slc26a3, little or no fluid secretory response). Another transporter that is expressed in a villous-predominant fashion is NHE3, whose pharmacological inhibition increases basal HCO_3^- secretory rates (Clarke *et al.* 2001; Repishti *et al.* 2001; Furukawa *et al.* 2004; Singh *et al.* 2010) in a predominantly Slc26a3-dependent fashion (Singh *et al.* 2010). We investigated the effect of NHE3 ablation (Fig. 6*A* and *B*) or pharmacological inhibition (Fig. 6*C* and *D*) on acid-induced DBS-R. NHE3 ablation or inhibition increased basal DBS, as would be expected from the inhibition of a transporter, which exports protons. The initial increase in acid-induced DBS-R was still seen, whereas no late acid-induced increase in DBS-R was observed. The results indicate that acid-induced DBS-R has two components, a fast, non-NHE3-dependent one, and a slower NHE3-dependent component, because the latter is seen in neither NHE3-deficient duodenum nor after pharmacological NHE3 inhibition.

Membrane trafficking of sodium/hydrogen exchanger isoform 3

The location of NHE3 in villous enterocytes during acid- and FSK-induced stimulation of the HCO_3^- secretory response was studied by immunohistochemistry. While FSK caused rapid NHE3 endocytosis (Fig. 7*B* and *E*), an acid pulse caused a biphasic trafficking of NHE3 (Fig. 7*C*–*E*). At 20 min after acid exposure, the amount of NHE3 in the BBM was higher than in control conditions (Fig. 7*C* and *E*), whereas it was lower than in controls at later time points (Fig. 7*D* and *E*). As the Slc26a3 and Slc26a9 antibody available to us is not specific for the respective isoform in the duodenum (possibly because of high expression levels of other Slc26 family members such as Slc26a6), trafficking studies for these transporters were not feasible. CFTR trafficking into the BBM upon acid exposure has been previously reported (Jakab *et al.* 2012)

SPAK, AKT and p38 phosphorylation is induced by forskolin as well as luminal acid contact in duodenal mucosa

SPAK and OSR1 phosphorylation are downstream signalling events of WNK activation (Delpire & Austin, 2010) that have been implicated in a change in anion selectivity of CFTR from Cl^- predominant to HCO_3^- predominant (Park *et al.* 2010), and have been shown to be activated by FSK in murine intestine (Xiao *et al.* 2012). Acid exposure also increased phosphoSPAK in duodenal mucosa, albeit not as strongly as FSK application (Fig. 8*A* and *B*). Immunohistochemistry localized WNK1, WNK4, SPAK and pSPAK predominantly to the basal cell pole of cryptal enterocytes in controls (Supplementary Fig. 2), and an increased intensity of all four proteins near the basal and an appearance in the apical cell pole upon FSK activation. All tested conditions, i.e. acid exposure, FSK and hypotonic Cl^- free solution increased the level of pSPAK+pOSR1 fluorescence signal compared to the saline perfusion (control) (Supplementary Fig. 3). Hypotonic Cl^- free condition was taken as positive control for the activation of WNK/SPAK pathway as previously has been shown by Park *et al.* (2010).

Secondly, we looked at AKT phosphorylation, which is a downstream signalling event of a number of kinases, including PI3 kinase (Hemmings & Restuccia, 2012). An increase in AKT phosphorylation at the tyrosine 308 phosphorylation site but not the Ser473 was seen

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immediately after luminal acid contact. FSK also resulted in an increase in phosphorylation of this site, albeit weaker than the acid (Fig. 8*A* and *B*). Hypotonic Cl^- free solution inhibited the phosphorylation of the AKT.

Thirdly, we studied p38 phosphorylation, also involved in NHE3 regulation (Shiue *et al.* 2005). p38 phosphorylation increased after acid and hypotonic Cl^- free solution, but not after FSK exposure (Fig. 8*A* and *B*). Immunohistochemistry localized p38 to the villus region, while pAKT (308) was localized to both crypt and villus (Supplementary Fig. 4).

Taken together, the results show that luminal acid pulse, as compared to FSK application, activates multiple overlapping but not identical signalling pathways in duodenal mucosa, with a differential distribution of these signalling events along the crypt–villus axis.

mRNA Expression of bicarbonate secretion-relevant transporters in Slc26a3- and Slc26a9-deficient and wild-type duodenum

To search for compensatory changes, we studied the mRNA expression of duodenal acid/base transporters involved in HCO_3^- secretion (see Seidler & Sjöblom, 2012 for review) in the Slc26a3-, Slc26a9- and

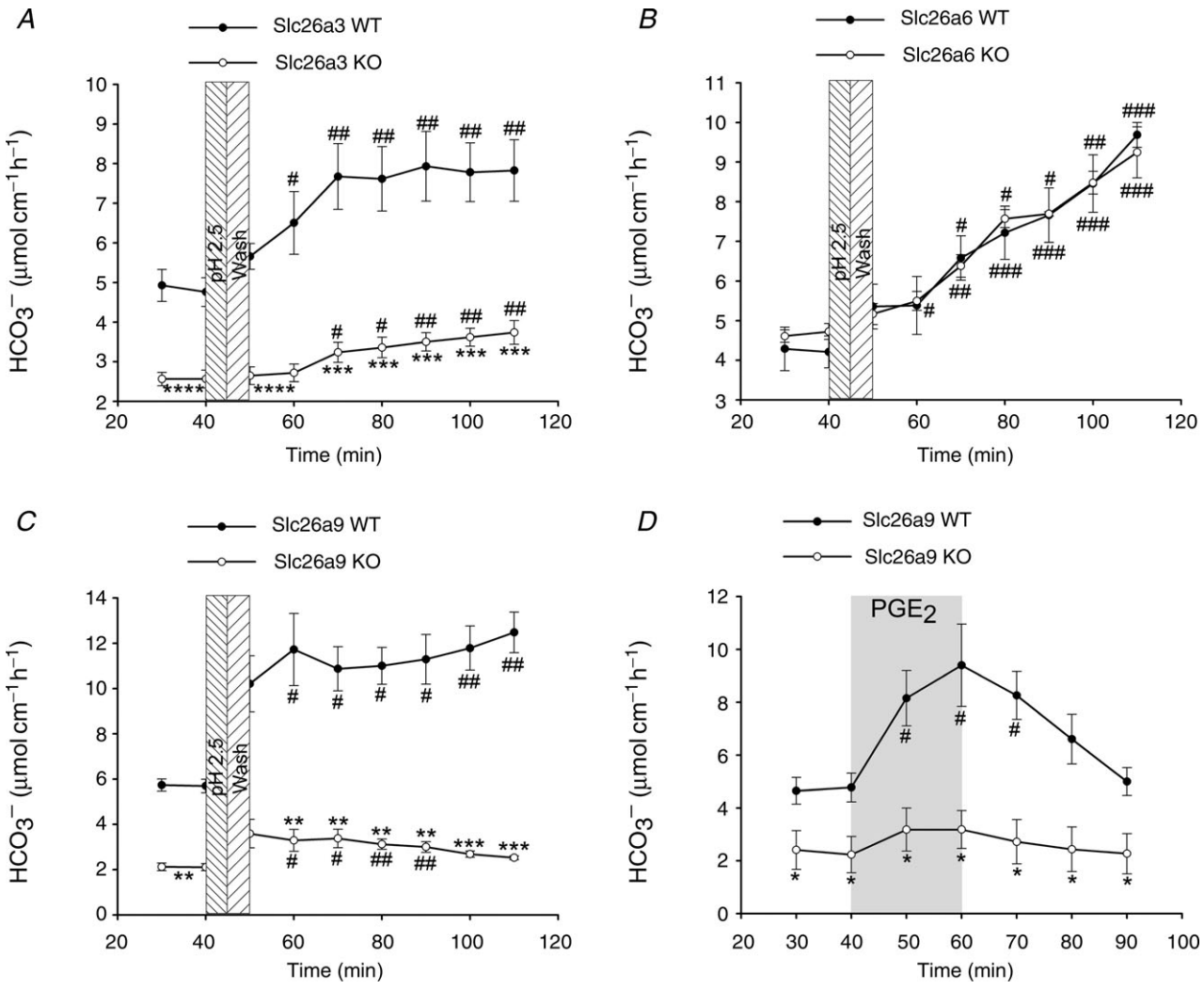

Figure 4. Basal DBS and acid-induced DBS-R in Slc26a3, a6 and a9 KO and WT mice
A, basal DBS as well as acid-induced DBS-R were significantly reduced in Slc26a3 KO compared to WT mice. *B*, neither basal DBS nor acid-induced DBS-R were different in Slc26a6 KO mice as compared to the WT littermates. *C*, both basal DBS as well as acid-induced DBS-R in Slc26a9 KO compared to WT mice were strongly reduced. *D*, both basal as well as 1 μM PGE_2-stimulated DBS-R were significantly reduced in Slc26a9 KO compared to WT duodenum. Values presented as means ± S.E.M. #Significant increase as compared to basal within the same group. *Significant decrease in KO mice as compared to their WT littermates. #, *$P < 0.05$; ##, **$P < 0.01$; ###, ***$P < 0.001$; ****$P < 0.0001$; $n = 4$–6. DBS-R, duodenal bicarbonate secretory response; KO, knockout; PGE_2, 16,16-Dimethyl Prostaglandin E_2; WT, wild-type.

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Slc9a3-deficient mice and their respective litter and gender-matched WT proximal duodenum controls. In the Slc26a9-deficient duodenum, no significant differences in CFTR, NBCn1/NBCe1, Slc26a3 and Slc26a6 mRNA expression were detected, but NHE3 mRNA expression was significantly decreased (Fig. 9*A*). CFTR appeared membrane-localized and crypt-predominant in the Slc26a9-deficient as well as WT duodenum (Supplementary Fig. 5). Immunohistochemical staining revealed NHE3 had microvillar localization but somewhat weaker in Slc26a9 KO than control duodenum (Supplementary Fig. 6). No differences in the mRNA levels of genes investigated were found in Slc26a3 as well as the Slc9a3 KO mouse (Fig. 9*B* and *C*). No compensatory increase in levels of Slc26a3 or Slc9a3 was seen in Slc26a6 KO mouse proximal jejunum (duodenum was not studied) (Supplementary Fig. 7).

Discussion

Luminal acid-induced HCO_3^- secretion is a biologically important, highly complex event, and neither the stimulatory pathways, nor the involved transporters and cellular origin of the secretion are fully understood (Allen & Flemstrom, 2005; Seidler & Sjöblom, 2012; Singh *et al.* 2012). The present study aimed to investigate further into the transport mechanisms and signalling pathways involved in acid-induced duodenal HCO_3^- secretory response.

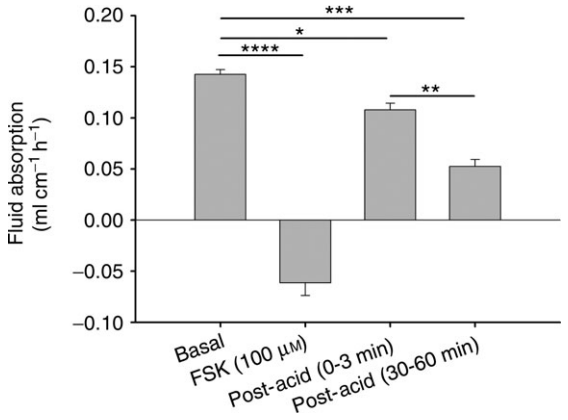

Figure 5. FSK- and acid-induced changes in fluid transport in the proximal duodenum of C57BL/6N mice
A change from fluid absorption under basal conditions (left bar) to fluid secretion was observed during application of 100 μM FSK (second bar). In contrast, the short acid pulse (pH 2.5 for 5 min) caused only a slight but significant reduction of fluid absorption in the first 30 min after the acid pulse (third bar), and a stronger reduction in the second 30 min (right bar). Values are presented as means ± S.E.M.*Significant differences between the bars connected with the horizontal line. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$; $n = 7$. FSK, forskolin.

First, it needs to be clarified what we call 'acid-induced HCO_3^- secretory response (DBS-R)' in this study: we measured the long-lasting stimulation of duodenal HCO_3^- secretion after a short (few minutes) exposure of the duodenal mucosa to a low pH (~2–3, depending on species). This stimulation involves axonal reflexes (Takeuchi *et al.* 1991) and the central nervous system (Singh *et al.* 2012) and can therefore only be studied *in vivo*. Long periods of acid exposure and/or low pH 'break' the duodenal barrier and cause passive paracellular leakage of alkaline interstitial fluid into the lumen (Vattay *et al.* 1988; Nylander *et al.* 1994). Previous studies clarified the optimal experimental conditions in murine duodenum *in vivo*, carefully avoiding barrier break (Singh *et al.* 2008).

The ion transport pathways involved in the 'physiological' acid-induced DBS-R are incompletely understood, except that CFTR expression is essential for a full acid-induced DBS-R (Hogan *et al.* 1997; Singh *et al.* 2008). This paper confirms and extends the findings in CFTR-deficient mice by showing that pharmacological inhibition of CFTR also results in a decreased HCO_3^- secretory response to acid.

The dependency of acid-induced DBS-R on the presence of luminal Cl^- (Fig. 2) suggested that luminal anion exchangers, which are probably members of the Slc26 gene family, are also necessary for acid-induced DBS-R. Because no selective inhibitors for the Slc26 members are available, we studied the KO mice for three Slc26 members with proven or potential Cl^-/HCO_3^- exchange properties known to date to be expressed in the duodenal BBM.

Slc26a3 (DRA)-deficient duodenum

Similar to the previous finding by Walker *et al.* (2009) in isolated murine duodenal mucosa, we found an almost 50% decrease in basal DBS in Slc26a3-deficient duodenum *in vivo*. However, the FSK-stimulated DBS-R was identical to WT. On the other hand, acid-stimulated DBS-R was strongly affected by Slc26a3 ablation. This indicates a major role for Slc26a3 in acid-induced HCO_3^- secretion in duodenal villous epithelium. A prominent duodenal ulcer phenotype has not been reported in patients with congenital chloride diarrhoea who carry mutations in the *Slc26a3* gene, but it is also not known whether gastric acid secretory rates are normal in these patients.

Slc26a6 (PAT-1)-deficient duodenum

Slc26a6 deletion, on the other hand, did not affect acid-induced DBS-R. Slc26a6 is expressed along the length of the duodenal villus possibly with some predilection for the villus tip region (Wang *et al.* 2002; Simpson

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et al. 2007). Some recent experimental data suggest a 2 HCO_3^-/1 Cl^- transport mode for Slc26a6, which would theoretically make it a strong HCO_3^- export mechanism in the duodenal villous enterocytes. Slc26a6-deficient isolated duodenal mucosa displayed a lower basal as well as PGE_2-stimulated, but not FSK-stimulated HCO_3^- secretory rate (Tuo *et al.* 2006), which can be also found *in vivo*, but the Slc26a6-mediated HCO_3^- secretory rate appeared to correlate with high systemic CO_2/HCO_3^- concentrations, although this was not rigorously tested (Singh *et al.* 2008). In a recent study, the duodenal tip enterocyte pH_i was assessed fluorometrically during and up to 25 min after a 5 min pH 2.5 acid pulse in exteriorized vascularly perfused murine duodenum, and the pH_i stayed below baseline for a prolonged period (Singh *et al.* 2013). Either the relatively low pH_i or an associated hypothetical membrane hyperpolarization, may be prohibitive for a contribution of Slc26a6 to acid-induced HCO_3^- secretion, but may affect an electroneutral (Alper *et al.* 2011) or 2:1 coupled (Shcheynikov *et al.* 2006) Slc26a3 to a lesser degree. Other possibilities may be acid-induced Slc26a6 internalization or inhibition, but we did not have antibody or selective inhibitors to study this question.

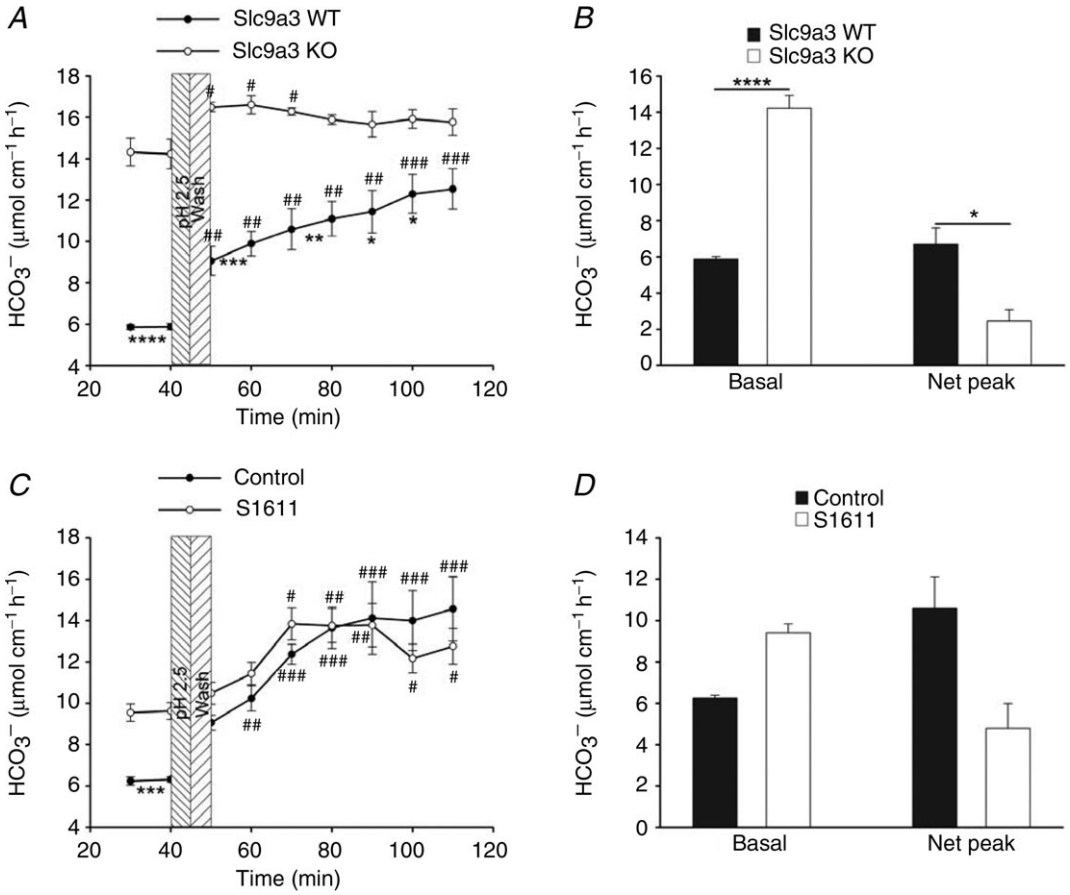

Figure 6. Pharmacological inhibition and genetic deletion of Slc9a3 increased basal DBS but blunted the late phase of acid-induced DBS-R

A, time course for basal DBS and acid-induced DBS-R in NHE3 (*Slc9a3*) KO and WT mice, and (C) with and without pharmacological inhibition by the selective NHE3 inhibitor S1611 (40 μM luminally, added at the beginning of the equilibration period). *B* and *D*, show the basal and acid-induced DBS-R for NHE3 KO and WT mice and during concomitant application of S1611. Both NHE3 deletion as well as NHE3 inhibition resulted in a significantly higher basal DBS (consistent with an active luminal Na^+/H^+ exchanger under basal conditions). The acid pulse still significantly increased DBS in the initial phase, but not in the late phase of the acid-induced DBS-R. Net peak response was calculated by subtracting the average basal value from the peak response, where peak response was taken as the maximal response of each experiment after the acid challenge. Values are presented as means ± S.E.M. #Significant increase as compared to the basal response within the same group. *Significant differences between the compared groups in the time course experiment and the bars connected with the horizontal line. #, $^*P < 0.05$; ##, $^{**}P < 0.01$; ###, $^{***}P < 0.001$; $^{****}P < 0.0001$; $n = 4$–5. DBS-R, duodenal bicarbonate secretory response; KO, knockout; NHE3, sodium/hydrogen exchanger isoform 3; WT, wild-type.

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Slc26a9-deficient duodenum

In contrast to Slc26a6 and Slc26a3, Slc26a9 expression in the duodenum is weak (Xu *et al.* 2005). The expression of human Slc26a9 or the murine analogue in HEK293 cells, as well as in *Xenopus* oocytes, suggested that Slc26a9 functions exclusively as a Cl^- channel (Dorwart *et al.* 2007), although other transport modes such as Cl^-/HCO_3^- exchange (Xu *et al.* 2005), or Na^+ coupled Cl^- channel or Cl^-/HCO_3^- exchanger (Chang *et al.* 2009) have been described. We recently found that Slc26a9 was responsible for the interleukin-13-induced anion conductance in murine bronchi (Anagnostopoulou *et al.* 2012). The magnitude of this current was identical in the presence and absence of CO_2/HCO_3^- in the perfusate, demonstrating that Slc26a9 can function as an HCO_3^- independent anion conductance. The results of the present study do not favour its function as a Cl^-/HCO_3^- exchanger because the low expression of Slc26a9 compared to Slc26a3 would not explain why the absence of either

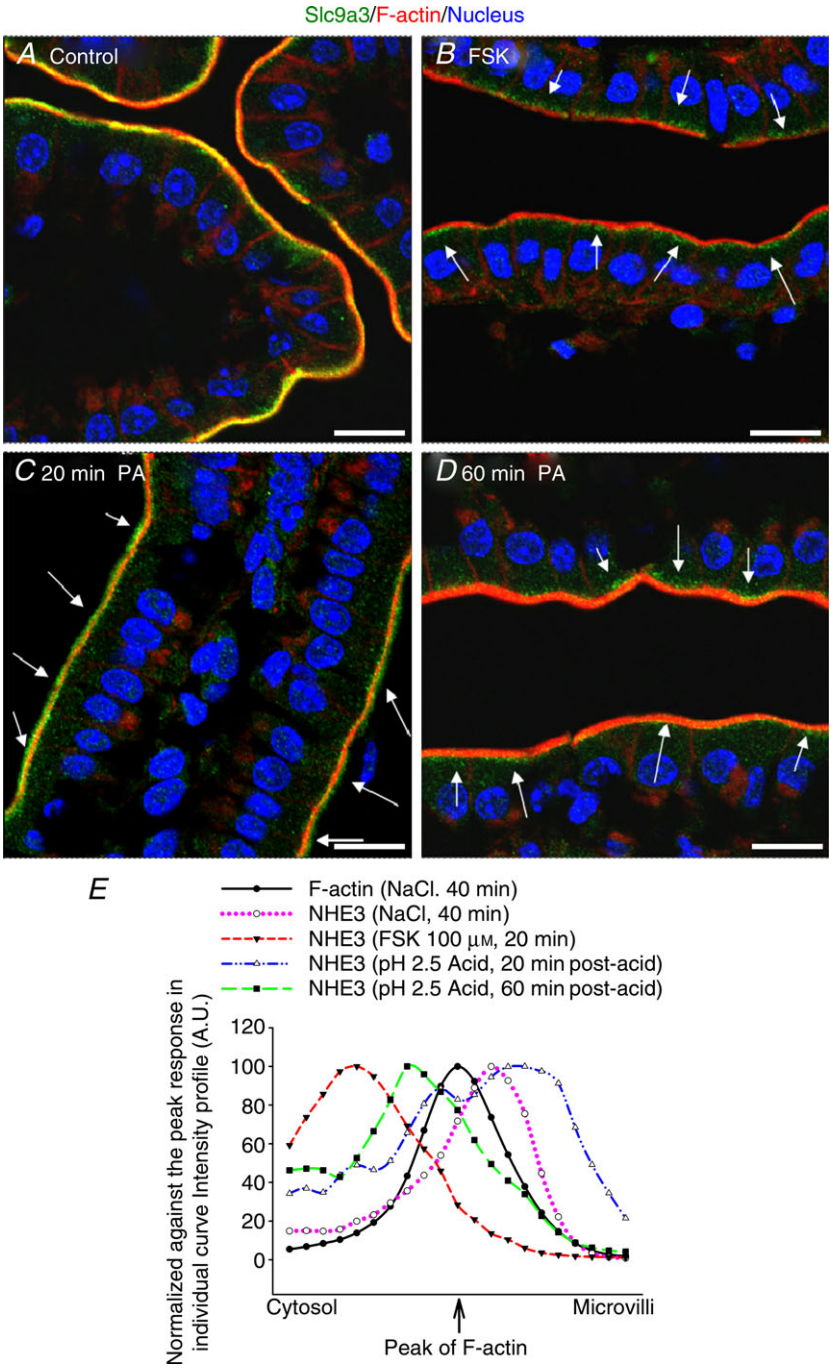

Figure 7. FSK- and acid-induced changes in NHE3 localization in the duodenal enterocyte brush border membrane
The control duodenum shows localization of NHE3 in the (*A* and *E*) microvillar region, as indicated by the peak of the NHE3 staining (*E*, pink curve) on the right (more extracellular) side of the F-actin (*E*, black curve, peak intensity of F-actin indicates the terminal web region; Chen *et al.* 2010). FSK (100 μM) treatment for 20 min shifted the NHE3 staining from the right side to the left (more intracellular) side of the peak intensity of F-actin staining, indicating internalization of NHE3 (*B* and *E*, red colour). Twenty minutes post-treatment with saline pH 2.5 for 5 min increased the amount of the NHE3 in the microvillar region as compared to the control (*D* and *E*, blue curve), whereas 60 min post acid pulse showed most of the NHE3 on the inner side of the terminal web region similar to that of the FSK (*D* and *E*, green colour). Scale bar 15 μm. Representative image out of three independent staining from three animals. FSK, forskolin; NHE3, sodium/hydrogen exchanger isoform 3; PA, tissue harvested at indicated time, 5 min post-acid treatment.

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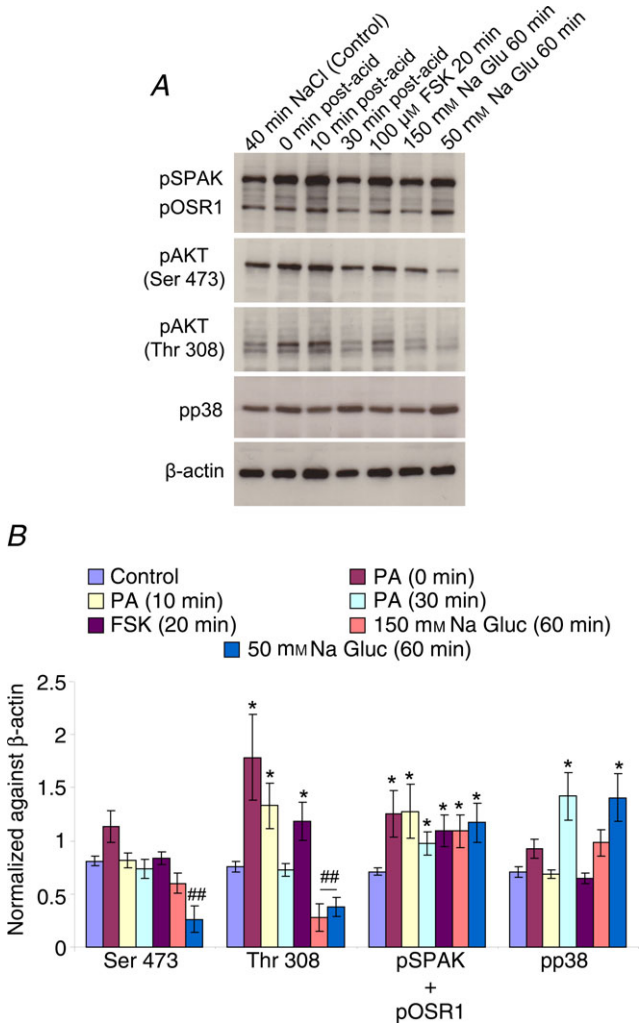

Figure 8. Forskolin and acid pulse induced different signalling pathways
A, representative Western blot for pSPAK/pOSR1, pAKT (Ser473), pAKT (Thr308) and pp38 after luminal perfusion of the proximal duodenum with different agents for the duration indicated in the figure. *B*, bars represent the quantification of the phosphoprotein bands in relation to the loading control from five individual experiments. pAKT (Ser473) significant increase in phosphorylation of AKT at Ser473 was seen with any treatment. pAKT (Thr308) of pAKT (Thr308) was induced strongly and rapidly by acid contact, also to some extent by FSK and was diminished by hypotonic sodium gluconate. pSPAK/pOSR1 FSK- and acid-induced phosphorylation of pSPAK/pOSR1. Cl^- free and hypotonic Cl^- free perfusions were used as positive controls (Park *et al.* 2010; Xiao *et al.* 2012). The same antibody detects the phosphorylation in the T-loop of both SPAK and OSR1. pp38 acid and hypotonic Cl^- free perfusions induced phosphorylation of p38, while FSK did not. Values are presented as means ± S.E.M. #Significant decrease compared to the control group. *Significant increase compared to the control group. The detailed procedure is explained in the Materials and methods section. A pairwise statistical comparison of bars with respect to their control has been provided in the supplementary file (Supplementary Fig. 9). FSK, forskolin; Na Glu, sodium gluconate; PA, tissue harvested at indicated time, 5 min post-acid treatment.

transporter has such a strong effect on basal as well as acid-induced DBS. CFTR expression was not lost in the absence of Slc26a9 (Fig. 9 and Supplementary Fig. 5). On the other hand, NHE3 expression was significantly downregulated in these mice, which appears to be a function of age, as we did not observe it in 6-week-old mice (Brigitte Riederer 2013 unpublished observations). This could explain some of the defective acid-induced HCO_3^- secretory response, but not the decrease in basal HCO_3^- secretion. The fact that FSK-induced DBS-R was abolished in CFTR-deficient mice, but preserved in Slc26a9-deficient mice argues against the notion that Slc26a9 tightly regulates duodenal CFTR function, as has been discussed based on results from cell lines (Bertrand *et al.* 2009; Avella *et al.* 2011). Our results do not rule out a modulatory role of Slc26a9 on CFTR, or vice versa, in a subset of cells. Another possibility is that Slc26a9 functions as a CFTR-dependent Cl^- conductance in the duodenum, and provides a Cl^- shunt pathway for Slc26a3-mediated Cl^-/HCO_3^- exchange. This hypothesis could explain the data, but is impossible for us to test at present.

We also wondered whether the early loss of the acid-secretory capacity in the stomach of Slc26a9-deficient mice, which has been described (Xu *et al.* 2008), may be the reason for a loss of acid-induced DBS-R, as well as the downregulation of NHE3 expression. However, this was not the case, as acid-induced DBS-R as well as duodenal NHE3 expression was preserved in two other mouse models with early loss of acid secretory capacity (KNCQ1-deficient as well as NHE2-deficient mice, Supplementary Fig. 8; Schultheis *et al.* 1998; Song *et al.* 2009). The role of Slc26a9 in the duodenum warrants further investigation.

Sodium/hydrogen exchanger isoform 3-deficient or -inhibited duodenum

An inhibition of NHE3 by Na^+/H^+ exchange inhibitors increases the duodenal DBS, probably because of the inhibition of proton secretion (Repishti *et al.* 2001; Furukawa *et al.* 2004). This increase in HCO_3^- output was dependent on and therefore probably mediated by Slc26a3, and it required CFTR function (Singh *et al.* 2010). We therefore hypothetized that the short acid pulse, via the release of neurotransmitters, may result in inhibition of NHE3. Surprisingly, it was not the early but the late increase in acid-induced DBS-R that was abolished by NHE3 inhibition or knockout. To better understand these findings on a molecular basis, we performed immunohistochemical staining and assessed the localization of NHE3 in relation to the BBM at different time points during the experiments. We observed a trafficking of NHE3 from the terminal web pool into the microvilli immediately after the acid pulse, followed by retrieval

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from the microvillar localization later on. A potential explanation is that the cellular acidification that occurs in the upper villous enterocytes during the short acid pulse (Singh *et al.* 2013) causes NHE3 trafficking into the membrane and proton extrusion, later followed by NHE3 inhibition and internalization due to acid-induced release of secretagogues that stimulate cAMP, cGMP, Ca^{2+}-dependent or possibly other signalling events in the enterocytes (see Allen & Flemstrom, 2005 and Seidler & Sjöblom, 2012 for review), and thus an increase in HCO_3^- output.

What could be the reasons for the strong dependency of acid but not FSK-induced DBS-R on Slc26a3 expression? NHE3 internalization in the villus epithelium occurs both during acid- as well as during FSK-induced DBS-R. This may leave Slc26a3-mediated HCO_3^- extrusion unmatched by proton extrusion, as we reported previously (Singh *et al.* 2010), as a major mechanism for alkalinizing the lumen. What then makes FSK, which also causes an even more rapid internalization of NHE3, stimulate a Slc26a3 independent as well as luminal Cl^- independent HCO_3^- secretory response? Under the assumption of differential signalling, possibly even with different predilections along the crypt–villus axis, in acid *vs.* FSK-induced DBR-R, we studied the fluid secretory response to FSK and acid pulse. Duodenal fluid secretion, in contrast to HCO_3^- secretion, is still considered predominantly of cryptal origin because the expression of the two transporters associated with a reduction in duodenal fluid secretion, namely NKCC1 and CFTR, is crypt predominant (Singh *et al.* 2010; Jakab *et al.* 2011, 2012). Indeed, a strong fluid secretory response was observed within minutes of FSK application, whereas an acid pulse resulted only in a slowly progressive inhibition of fluid absorption over the full observation period (Fig. 5). This suggested that differential signalling might occur along the crypt–villus axis in acid- and FSK-stimulated duodenal HCO_3^- secretion.

We next searched for indications of this differential signalling. We had previously observed a strong activation of the WNK kinase pathway by FSK, resulting in phosphorylation of the downstream kinases SPAK and OSR1 (Xiao *et al.* 2012). This signalling event is associated with a change in anion selectivity of the CFTR channel from Cl^- to HCO_3^- predominance in pancreatic epithelial cells, as well as an inhibition of Cl^-/HCO_3^- exchange in pancreatic duct cells (Park *et al.* 2010). One hypothesis to explain our findings was that FSK, but not a short acid pulse, activates the WNK signalling pathway and renders HCO_3^- secretory response independent of luminal Cl^-/HCO_3^- exchangers. However, an increase in SPAK phosphorylation in a crypt-predominant localization was also observed 20 min after a short-term acid pulse (Supplementary Fig. 3).

PI3Kinase activation, with AKT phosphorylation as a downstream signalling event, has been associated with Slc26a3 (Saksena *et al.* 2008; Lissner *et al.* 2010) as well as NHE3 (Musch *et al.* 2009) transport activation through trafficking into the membrane. We therefore studied phosphoAKT expression in the duodenal mucosa, as well as AKT phosphorylation at Ser473 and Thr308

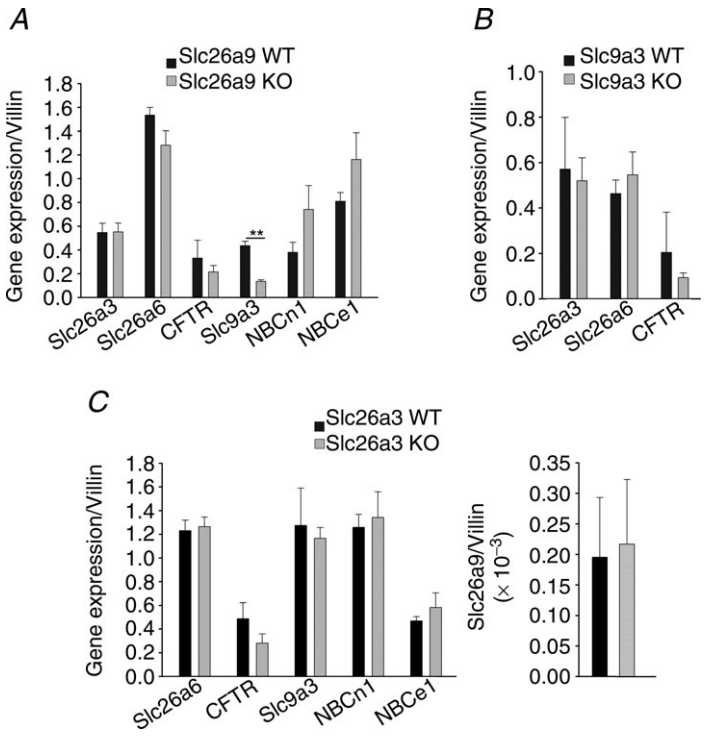

Figure 9. Transporter mRNA expression in the Slc26a3, Slc26a9 and Slc9a3 KO compared to WT duodenum
A, transporter mRNA expressions (with the brush border membrane protein villin as the control gene) in Slc26a9 KO *vs.* WT duodenum. The only differentially expressed transporter was the apical NHE3 (Slc9a3), which was significantly downregulated in the Slc26a9 KO duodenum of these relatively aged mice (3–5 months old) that were also used in the functional experiments (also see immunohistochemical staining in Supplementary Fig. 6), while NHE3 mRNA expression was not altered in 6 week old mice (data not shown). *B*, transporter mRNA expression in Slc9a3 KO and WT duodenum. No significant difference was found for any of the tested genes and similar results were obtained for Slc26a3 KO and WT duodenum (*C*). ($n = 5$). CFTR, cystic fibrosis transmembrane conductance regulator; KO, knockout; NHE3, sodium/hydrogen exchanger isoform 3; WT, wild-type.

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during acid and FSK-induced duodenal HCO_3^- secretory response *in vivo*, by Western analysis of scraped duodenal mucosa. A very rapid increase in AKT phosphorylation at Thr308 was seen after contact of the mucosa with acid, but a lesser increase in Thr308 phosphorylation was also seen after FSK stimulation. Luminal Cl^- removal and hypotonicity strongly decreased the amount of phosphorylated AKT both at Ser473 and Thr308. p38 phosphorylation has also been associated with translocation of NHE3 into the membrane (Shiue *et al.* 2005), although no information exists about its regulation of the Slc26 family members. Interestingly, p38 phosphorylation occurred in a villus-predominant fashion and was activated only by luminal acid, not by FSK. Luminal hypotonicity, which also increases duodenal anion secretion (Nylander *et al.* 2003), caused a strong increase in p38 as well as SPAK/OSR1 phosphorylation, while decreasing phosphoAKT. The experiments therefore support the concept that multiple signalling events occur along the crypt–villus axis with a differential time and spatial fashion in acid *vs*. FSK-induced HCO_3^- secretion. Because the application of pathway inhibitors in these *in vivo* perfusion studies is not likely to yield interpretable results, we did not attempt to study the causality of these signalling events to the observed changes in HCO_3^- secretion.

In summary, our study of the ion transporters and potential regulatory pathways involved in acid-induced duodenal HCO_3^- secretory response *in vivo* provides insight into a highly complex interplay of different acid–base transporters and different signalling pathways that dominate the HCO_3^- secretory rate at different time points and with spatial segregation along the crypt–villus axis following the acid pulse. The result is an increase in HCO_3^- secretory rate long after the initial contact of the duodenal mucosa with acid. Our study firmly establishes the previously unrecognized involvement of Slc26a3, Slc26a9 and NHE3 in the acid-induced HCO_3^- secretory response. As Slc26a3 is directly inhibited by non-steroidal anti-inflammatory drugs (Chernova *et al.* 2003), as Slc26a9 is directly inhibited by ammonia (Xu *et al.* 2005) and as NHE3 is subject to regulation by a variety of commonly used cardiovascular medications, the causality of the high incidence of complicated duodenal ulcer disease at advanced age should be revisited.

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Additional information

Competing interests

None.

Author contributions

A.K.S., Y.L., B.R., R.E. and U.S. designed, performed and analysed experiments, B.K.T. and M.S. provided expert assistance and suggestions, and A.K.S and U.S. wrote the manuscript. The authors have no conflict of interest to disclose. All the authors approved the final version of the manuscript.

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