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Article

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Na⁺-dependent transporters mediate HCO₃⁻ salvage across the luminal membrane of the main pancreatic duct

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Introduction

Pancreatic ducts serve a secretory function to produce a fluid rich in Na⁺ and HCO₃⁻ in response to secretin stimulation, and also function as a conduit to carry pancreatic enzyme and fluid secretions to the intestine (1). Secretin-stimulated HCO₃⁻ secretion across the luminal membrane (LM) requires H⁺ secretion or HCO₃⁻ absorption across the basolateral membrane (BLM) to maintain constant cytosolic pH (pH_i) and replenish the secreted HCO₃⁻. Current evidence suggests that a Na⁺ and HCO₃⁻ cotransporter mediates most HCO₃⁻ influx across the BLM (2, 3). A Na⁺/H⁺ exchanger (NHE) in the BLM is assumed to help HCO₃⁻ secretion by controlling pH_i to prevent intracellular acidification during HCO₃⁻ secretion (4). Because the pancreatic duct secretes HCO₃⁻ and Na⁺, NHEs are not expected to be present in the LM. However, using perfused ducts, we and others have reported NHE activity in the LM of pancreatic ducts of different species (4, 5). The proteins mediating this activity, their regulation, and their physiological significance are unknown.

NHEs are a family of membrane proteins involved in regulation of pH_i, cell volume, and electrolyte transport. Since the initial cloning of NHE1 (6), at least six NHE iso-

forms have been found in mammalian cells; five of these were identified in rodent tissue (7). The physiological role of only a few of the isoforms has been established with any certainty. NHE1 is ubiquitously expressed, and has a housekeeping role (8). NHE1 is always expressed in the BLM of epithelial cells (9). In many epithelia, such as salivary glands, small intestine, colon, and kidney, NHE2 and NHE3 are the major isoforms expressed in the LM (7, 9–11). In the kidney, NHE3 in the proximal tubule mediates 60% of Na⁺ and HCO₃⁻ absorption (12, 13). NHE2 is believed to mediate a large portion of Na⁺ absorption in the small intestine (9). The phenotype of a targeted *NHE3* knockout mouse supports a major role for NHE3 in renal Na⁺ and HCO₃⁻ absorption (14). However, based on the phenotype of the *NHE2*^{-/-} mouse, so far the function of NHE2 appears to be essential only in gastric parietal cells (15). The role of NHE2 and NHE3 in other tissues that express these proteins is not known.

The availability of *NHE2*^{-/-} and *NHE3*^{-/-} animals provided an opportunity to clarify the role of these NHE isoforms and other H⁺ efflux mechanisms in the HCO₃⁻-secreting pancreatic duct. We find that NHE1 is expressed in the BLM, whereas NHE2 and NHE3 are expressed in the LM. Furthermore, we found a novel

amiloride-sensitive, Na⁺-dependent H⁺ efflux (or HCO₃⁻ influx) mechanism that does not correspond to any known NHE isoform. The use of ducts from wild-type (WT), *NHE2*^{-/-}, and *NHE3*^{-/-} mice showed that NHE3 was responsible for about 45% of the luminal H⁺ efflux, and the novel mechanism for about 55%. Despite the expression of NHE2 in the LM, no functional role for this isoform could be established. Luminal Na⁺-dependent H⁺ efflux mechanisms were decreased by cAMP. Based on these findings, we proposed a role for luminal Na⁺-dependent H⁺ efflux mechanisms in controlling electrolyte composition of the pancreatic juice during basal pancreatic fluid secretion.

Methods

2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethylester (BCECF-AM) was purchased from Molecular Probes Inc., Eugene, Oregon, USA. HOE 694 was a gift from Hans Lang (Hoechst AG, Frankfurt, Germany). Antibody recognizing NHE1 (16) was a gift from Sergio Grinstein (Hospital for Sick Children, Toronto, Canada). Antibody specific for NHE2 (17) was a gift from Eugene Chang (University of Chicago, Chicago, Illinois, USA), and antibody specific for NHE3 (12) was a gift from Orson Moe, University of Texas Southwestern Medical Center, Dallas, Texas, USA.

The standard perfusate was termed pancreatic solution A, and contained (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES (pH 7.4 with NaOH), and 10 glucose. Na⁺-free solutions were prepared by replacing Na⁺ with *N*-methyl-D-glucamine.

Animals and preparation of ducts. *NHE2* gene-disrupted (*NHE2*^{-/-}) or *NHE3* gene-disrupted (*NHE3*^{-/-}) mice were maintained on a standard diet. Genotyping was carried out on day 14 postpartum as described previously (14, 15). Gross microscopic examination did not reveal any apparent phenotypic abnormalities in the pancreas of the *NHE2*^{-/-} mice. The *NHE3*^{-/-} mice tended to be smaller than their WT or heterozygote littermates, which may have been a result of their chronic diarrhea (14). Accordingly, their pancreas also appeared smaller, but they otherwise had no discernible differences as judged by microscopic examination.

The procedure for preparation and perfusion of the main pancreatic duct was identical to that described previously (18). The animals were anesthetized by intramuscular injections of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The abdomen was opened and the entry of the common pancreaticobiliary duct was identified. The lumen was cannulated using a modified 31-gauge needle (TSK Stri-Ject; Air-Tite Products Co., Virginia Beach, Virginia, USA). After the cannula was secured and the proximal end of the common duct was ligated, the entire pancreas was removed to a dish containing ice-cold pancreatic solution A, to which 0.02% soybean trypsin inhibitor and 0.1% BSA had been added. The main duct was cleared of acini and connective tissue, and the proximal end of the main duct was cut to facilitate retrograde luminal perfusion.

Measurement of pH_i. The microdissected main duct was transferred to a perfusion chamber placed on the stage of an inverted microscope equipped with a fluorescence measuring system (Delta Ram; Photon Technology International Inc., South Brunswick, New Jersey, USA). The bath was perfused at a flow rate of 6 mL/min, and the lumen was perfused at a flow rate of 150 μL/min using pancreatic solution A at 37°C. The pH-sensitive fluorescent dye BCECF-AM was loaded through the luminal perfusate at a concentration of 2 μM in pancreatic solution A for 5 minutes. Dye loading was monitored at excitation wavelengths of 490 nm and 440 nm and an emission wavelength of 525 nm. After completion of dye loading, pH_i measurements were performed according to the specified protocols, and the fluorescence ratios at 490 nm and 440 nm were calibrated intracellularly by perfusing the cells with solutions containing 145 mM KCl, 10 mM HEPES, and 5 μM nigericin, with pH adjusted to 6.2–7.6, as described previously (10). Buffer capacity was calculated by measuring ΔpH_i in response to 5–20 mM NH₄Cl pulses (19).

RT-PCR. RT-PCR analysis was performed with rat pancreas rather than mouse pancreas, because the sequences of all the known NHE isoforms are available only for this species. To avoid contamination with other cell types, the rat pancreatic duct was carefully microdissected and cleaned of adherent cells. RNA was extracted from the microdissected ducts using a guanidinium thiocyanate-phenol chloroform solution (TRIzol; GIBCO BRL, Rockville, Maryland, USA), and was reverse transcribed using random hexamer primers and RNase H⁻ reverse transcriptase (GIBCO BRL). The cDNA was amplified using specific primers and AmpliTaq Gold enzyme (PE Biosystems, Foster City, California, USA); the products were separated on a 1.5% agarose gel containing 0.1 μg/mL ethidium bromide. Primers were selected from cDNA sequences of rat NHE1–5 isoforms (7, 10). The primer sequences were as follows:

β-actin, sense: 5'-CCC TAG ACT TCG AGC AAG AGA TGG-CCA CTG-3', antisense: 5'-CGG ATG TCA ACG TCA CAC TTC-ATG ATG GAA-3', size of PCR product: 211 bp;

rNHE1, sense: 5'-CCT TTC TGG GGT TTA CAC GGG AGG-GAC TGT-3', antisense: 5'-GTG GAG CTC TGA CTG GCA-GGG AAG ATT CAA-3', size of PCR product: 160 bp;

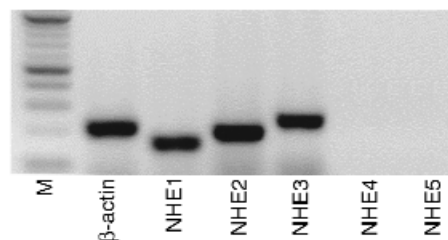


Figure 1

RT-PCR analysis of NHE isoforms in pancreatic ducts. The rat pancreatic duct expresses mRNA for β-actin (211 bp), NHE1 (160 bp), NHE2 (196 bp), and NHE3 (243 bp), but not for NHE4 and NHE5. The identity of each of the PCR-amplified products was confirmed by sequencing.

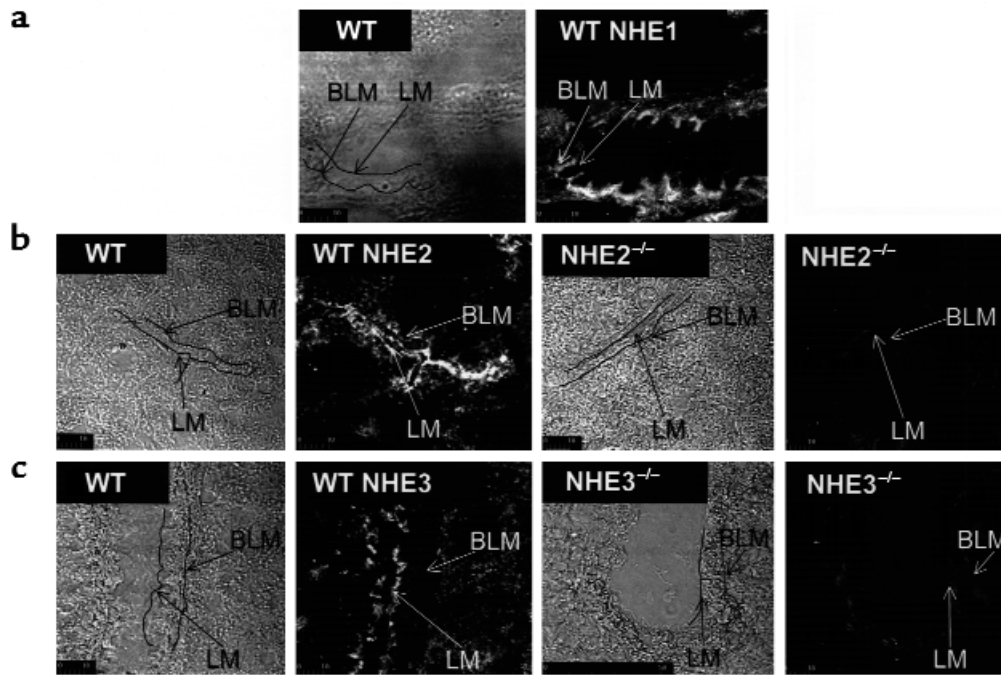


Figure 2

Immunolocalization of NHE isoforms in murine pancreas. Pancreatic sections were prepared from the indicated mouse lines and then stained with antibodies specific for NHE1 (a), NHE2 (b), and NHE3 (c). In each image pair, the first image is the phase contrast and the second image is the corresponding fluorescent image. Because it is difficult to visualize the sparse ducts in the pancreas that are embedded in the acinar structures without a counterstain, and because counterstaining is not compatible with immunofluorescence, the luminal (LM) and basal (BLM) borders are highlighted by black lines and marked by arrows in all phase-contrast images.

rNHE2, sense: 5'-TGA CGG TAT TAG GGC ACA GGT TGG-AAT GTA-3', antisense: 5'-AAA TTG GGA CAG AGG CGG-GGG TAA G-3', size of PCR product: 196 bp;

rNHE3, sense: 5'-AGG GAG ATC GAG ATG GGG CTA AAG-GTG GAC-3', antisense: 5'-AAG CAG ATG CAG TAT GTT GGG-CGG ACT TG-3', size of PCR product: 243 bp;

rNHE4, sense: 5'-TCT GAG GGT AGG GAT GAT TAA TTG-GTC ACA-3', antisense: 5'-GCA TTG GCC TGT TTC AAC ATT-TCT GA-3', size of PCR product: 126 bp;

rNHE5, sense: 5'-GGC CGA GAC CGA GAG GAT GTG-3', antisense: 5'-CGG GAT TAG CCA CAC CAT CCT TCT-3', size of PCR product: 244 bp.

Positive controls for NHE1, NHE2, and NHE3 were obtained from submandibular gland mRNA. Positive controls for NHE4 came from kidney mRNA, and positive controls for NHE5 were from brain mRNA. The identities of all amplified products were verified by nucleotide sequencing.

Immunohistochemistry. The pancreas from WT mice was embedded in OCT (Miles Inc., Elkhart, Indiana, USA), frozen in liquid nitrogen, and cut into 4- μ m sections. Pancreatic slices from *NHE2*^{-/-} or *NHE3*^{-/-} mice were also used as negative controls for the respective antibodies and to obtain conclusive evidence for the expression of each isoform. Immunostaining of frozen sections was done as reported previously (10). In preliminary experiments, we tested several tissue fixation, permeabilization, and staining procedures. All harsh fixation procedures eliminated recognition of the NHE isoforms by the respective antibodies; therefore the sections were

fixed and permeabilized by incubation with cold methanol for 10 minutes at -20°C. After removal of methanol, the slices were washed with PBS twice, and the tissue area was encircled using a hydrophobic marker (Pap Pen; Zymed Laboratories Inc., South San Francisco, California, USA). The nonspecific sites were blocked by incubation for 1 hour at room temperature with 0.1 mL PBS containing 5% goat serum, 1% BSA, and 0.1% gelatin (blocking medium). The medium was aspirated and then replaced with 50 μ L of blocking medium containing control serum and a 1:100 dilution of each antibody. After incubation with the primary antibody for 1.5 hours at room temperature, and three washes with PBS, the antibodies were detected with anti-rabbit goat IgG tagged with FITC (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). Images were collected with a confocal microscope (MRC 1024; Bio-Rad Laboratories Inc., Hercules, California, USA).

Data presentation. Data is presented as mean \pm SE for the indicated number of observations (where appropriate). Statistical analysis was determined using ANOVA. *P* < 0.05 was considered statistically significant.

Results

Identification of NHE isoforms in the pancreatic duct. The identities of the NHE isoforms expressed in the pancreatic duct were first analyzed by RT-PCR. Although secretory mechanisms may be slightly different between the mouse and the rat pancreatic ducts, we performed RT-PCR on rat mRNA because sequence data specific for all NHE iso-

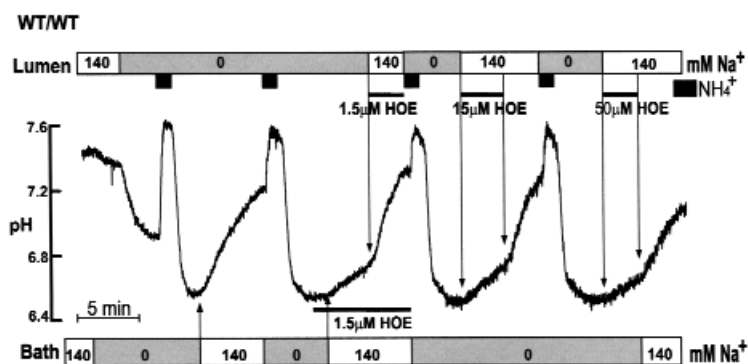


Figure 3

Na⁺-dependent H⁺ efflux in the BLM and LM of the pancreatic duct. NHE activity was measured in the main pancreatic duct. After NH₄⁺-induced acidification, the BLM was perfused with a Na⁺-containing solution with or without 1.5 μM HOE 694, as indicated. Subsequently, the LM was perfused with a Na⁺-containing solution supplemented with the indicated concentration of HOE 694.

forms are available only for the rat. To circumvent contamination by other cell types, acinar cells and vessel structures were removed from the main and interlobular ducts under microscopic inspection, and the ducts were washed twice with fresh pancreatic solution A. Figure 1 shows that the pancreatic duct expresses mRNA for NHE1, NHE2, and NHE3, but not for NHE4 and NHE5. The ability of the primers for NHE4 and NHE5 to detect the respective isoforms was verified using mRNA prepared from kidney and brain, respectively (data not shown). The identity of each of the PCR-amplified products was confirmed by nucleotide sequencing.

Next, we used immunostaining in an attempt to determine localization of the NHE isoforms. The results obtained with the mouse pancreas are shown in Figure 2. The staining with anti-NHE1 antibody in Figure 2a shows clear basolateral localization and an absence of luminal staining. However, because more than 95% of the plasma membrane is BLM, in many ducts staining with NHE1 appeared to encircle the cells, with occasional absence of staining in luminal borders. The polyclonal antibody recognizing NHE2 stained the LM of the duct (Figure 2b); the specificity of staining was verified by the absence of a significant signal in pancreatic sections obtained from *NHE2*^{-/-} mice. Expression of NHE3 was localized to the LM of duct cells (Figure 2c), and the speci-

ficity of the staining with this antibody was verified using the pancreas from *NHE3*^{-/-} mice. Sections from *NHE2*^{-/-} mice showed clear luminal NHE3 staining; those from *NHE3*^{-/-} mice showed clear luminal NHE2 staining (data not shown). However, the signal/noise was not sufficient to determine whether there was any compensatory increment of NHE expression in the pancreatic ducts of either mutant mouse.

Previously, it was shown that both the LM and BLM of the rat pancreatic duct express functional Na⁺-dependent H⁺ efflux activity, which was inhibited by 0.5 mM amiloride (4). Similar results were found in the mouse pancreatic duct (Figure 3). Resting pH_i of WT pancreatic duct cells was 7.38 ± 0.04 (*n* = 5) in the absence of HCO₃⁻. Perfusing the bath with a Na⁺-free medium caused a small decrease in pH_i, whereas perfusing both the bath and the lumen with Na⁺-free medium caused rapid and intensive acidification. The cells were further acidified by a luminal application of 20 mM NH₄Cl for 1–3 minutes. After removal of NH₄⁺, pH_i stabilized at 6.4–6.5. Application of Na⁺-containing solution to the bath caused rapid pH_i recovery. In five experiments, HOE 694 at 1.5 μM inhibited the Na⁺-dependent pH_i recovery by an average of 87% (see Table 1). Among the known NHE isoforms, only the *K_i* value for inhibition of NHE1 by HOE 694 is below 1.5 μM (20). Hence, the functional data substantiates the immunolocalization of NHE1 in the BLM. Subsequent application of a medium containing Na⁺ and 1.5 μM HOE 694 to the lumen showed that the luminal transporters have a lower affinity for HOE 694 than for the BLM NHE (Figure 3). Multiple experiments summarized in Table 1 showed that HOE 694 inhibited luminal NHE activity by 78% at 15 μM, and by 86% at 50 μM.

Identification of the NHE isoform mediating luminal Na⁺/H⁺ exchange activity. RT-PCR analysis and immunolocalization studies suggested the expression of NHE2 and NHE3 in the LM of the mouse pancreatic duct. Because inhibition of NHE3 in model systems required a high concentration of HOE 694 (*K_i* of 0.65 mM), and 50 μM HOE 694 had no effect on luminal NHE activity

Table 1

Inhibition of Na⁺-dependent pH_i recovery by HOE 694

Membrane	HOE 694 (μM)	Inhibition (%)
Basolateral	1.5	87 ± 7 (<i>n</i> = 5)
Luminal	1.5	8 ± 3 (<i>n</i> = 5)
Luminal	15.0	78 ± 4 (<i>n</i> = 5)
Luminal	50.0	86 ± 3 (<i>n</i> = 4)

Na⁺-dependent H⁺ efflux was measured as described in the legend to Figure 3, by applying 140 mM Na⁺ and varying concentrations of HOE 694 to either the luminal or basal membranes of acidified ducts. The other membrane was perfused with a Na⁺-free solution. The percent inhibition was calculated from the first derivative of the changes in pH_i.

in the mouse proximal tubule (21), we expected NHE2 to be the active isoform in the LM of the pancreatic duct (9, 20). To obtain unequivocal evidence for the role of each NHE isoform, we compared NHE activity in pancreatic ducts from WT, *NHE2*^{-/-}, and *NHE3*^{-/-} mice. Figure 4a shows representative traces from each mouse line; Figure 4b shows the summary of at least four experiments under each condition. The rate of luminal Na⁺-dependent pH_i recovery at an initial pH_i of 6.4–6.5 in ducts from WT mice was 0.93 ± 0.08 pH units/min (n = 5). Surprisingly, the rate of luminal Na⁺-dependent H⁺ efflux in pancreatic ducts from *NHE2*^{-/-} mice was 0.86 ± 0.09 pH units/min (n = 4), which was not significantly different from that in WT mice. By contrast, the rate of luminal Na⁺-dependent pH_i recovery was significantly reduced in ducts from *NHE3*^{-/-} mice (0.51 ± 0.02 pH units/min; n = 6). When interpreting these results, it is important to consider the buffer capacity of duct cells in all mouse lines. An increase in buffer capacity in ducts from *NHE3*^{-/-} mice could explain the reduced rate of Na⁺-dependent pH_i recovery. To exclude this possibility, the buffer capacity of duct cells from WT and *NHE* knockout mice was measured. Table 2 shows that there were no significant differences between the buffer capacity of duct cells from any of these mouse lines.

Of note is the remaining 55% of Na⁺-dependent H⁺ efflux in the LM of ducts from *NHE3*^{-/-} mice, and the normal activity in ducts from *NHE2*^{-/-} mice. Therefore, it was of interest to determine whether these activities could still be inhibited by HOE 694. Figure 5 shows that 50 μM HOE 694 inhibited the luminal Na⁺-dependent H⁺ efflux by more than 85% in ducts from *NHE2*^{-/-} and *NHE3*^{-/-} mice. Similar results were obtained with three ducts from each knockout line. Hence, it appears that a novel Na⁺-dependent and HOE 694-sensitive mechanism mediates 55% of luminal H⁺ efflux (or HCO₃⁻ influx) in the mouse pancreatic duct.

Regulation of luminal H⁺ efflux by cAMP. The main function of pancreatic duct cells is secretion of an HCO₃⁻-rich fluid in response to feeding stimuli such as secretin (22).

Having found at least two luminal Na⁺-dependent H⁺ efflux mechanisms, we proceeded to determine their possible regulation by the secretin-generated, second-messenger cAMP. After measuring luminal NHE activity under basal conditions, duct cells were treated with 5 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX) for 5 minutes (Figure 6a). Stimulation with forskolin and IBMX reduced luminal Na⁺-dependent H⁺ efflux in ducts from WT mice by 43 ± 5% (n = 5) (Figure 6b). This protocol was also performed with ducts from *NHE2*^{-/-} and *NHE3*^{-/-} mice. Inhibition by forskolin and IBMX in *NHE2*^{-/-} mice (45 ± 2%; n = 4) was not different from that measured in WT mice. The residual activity in ducts from *NHE3*^{-/-} mice was also inhibited by cAMP stimulation, although the inhibition was only 23 ± 6% (n = 6). Table 2 shows that inhibition of luminal Na⁺-dependent H⁺ efflux by cAMP was not due to a change in buffer capacity, because stimulation with forskolin and IBMX did not change cytoplasmic buffer capacity of pancreatic ducts from any of the lines. The effect of cAMP stimulation on BLM NHE was also investigated. In five experiments, the BLM NHE activity was not affected by forskolin and IBMX treatment (data not shown).

Discussion

The major aim of this study was to identify the Na⁺-dependent H⁺ secretory mechanisms in the LM of the pancreatic duct, and their possible roles in ductal physiology. Immunohistochemistry, backed by RT-PCR analysis, suggests expression of NHE2 and NHE3 in the LM of pancreatic duct cells. This is confirmed by the elimination of staining from the ducts of the respective knockout mice. The duct also expressed NHE1, which could be localized by a functional assay (sensitivity to HOE 694) and immunostaining to the BLM. Therefore, we can conclude that pancreatic ducts express NHE2 and NHE3 in the LM, and NHE1 in the BLM.

Functional analysis of Na⁺-dependent H⁺ transport in the LM of ducts from WT, *NHE2*^{-/-}, and *NHE3*^{-/-} mice revealed several surprises. The first unexpected finding

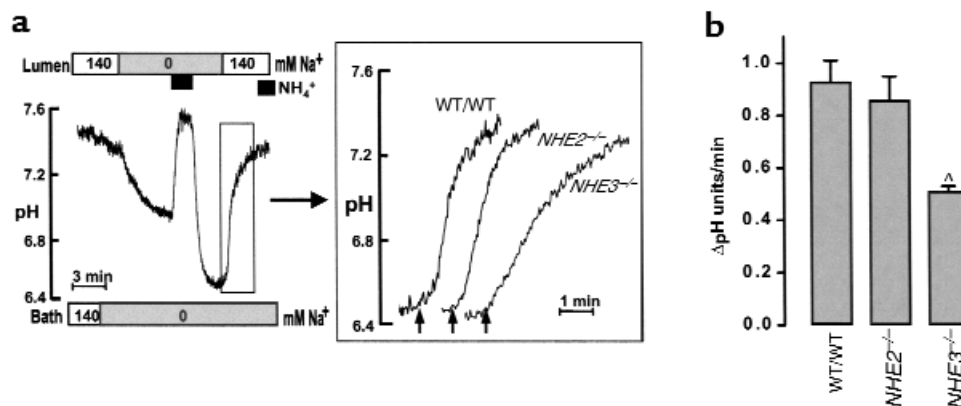


Figure 4

Luminal Na⁺-dependent H⁺ efflux in pancreatic ducts of WT, *NHE2*^{-/-}, and *NHE3*^{-/-} mice. Luminal Na⁺-dependent H⁺ efflux was measured in ducts acidified to the same level using the indicated protocol. (a) Examples of individual traces from each mouse line. (b) Summary of five, four, and six experiments performed with ducts from WT, *NHE2*^{-/-}, and *NHE3*^{-/-} mice, respectively. ΔP < 0.05 compared with WT.

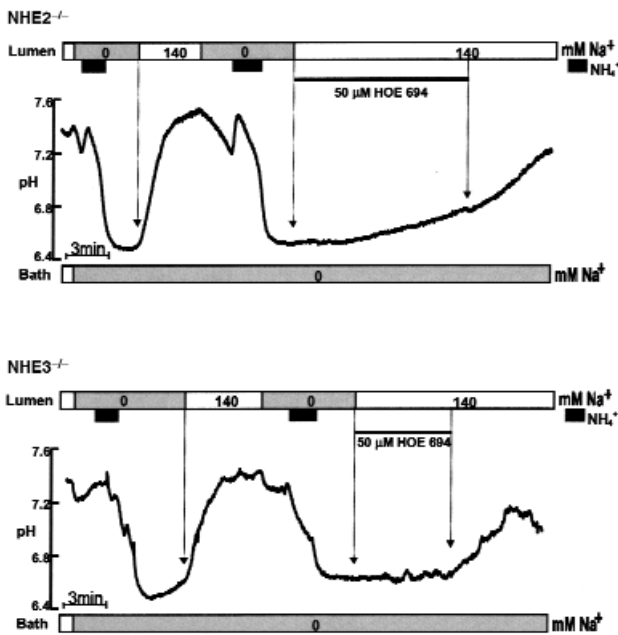


Figure 5
The luminal Na⁺-dependent H⁺ efflux is inhibited by HOE 694. The protocol outlined in the legend to Figure 3 was used to measure inhibition of luminal Na⁺-dependent H⁺ efflux in pancreatic ducts from *NHE2*^{-/-} (upper trace) and *NHE3*^{-/-} (lower trace) mice.

was normal luminal Na⁺-dependent H⁺ efflux in ducts from *NHE2*^{-/-} mice. From the sensitivity of the activity to HOE 694 (Table 1) and the reported *K_i* of the different NHE isoforms for inhibition by HOE 694 (20), we expected that most luminal H⁺ efflux would be mediated by NHE2. Several findings confirmed disruption of the *NHE2* gene in the knockout mice. The mice lacked mRNA coding for WT NHE2, and displayed severe histopathology of the gastric mucosa and a lack of acid secretory function (15). Furthermore, the NHE2 protein was absent from the parotid gland (23), the kidney (21), and the pancreas (this study). Yet, deletion of NHE2 protein had no effect on luminal Na⁺-dependent H⁺ efflux in pancreatic duct. Several explanations can account for this finding. Upregulation of other Na⁺-dependent transporters might have offset the effect of deletion of NHE2. A prediction of this scenario is that the residual activity in the LM of ducts from *NHE2*^{-/-} mice will show different HOE 694 sensitivity than that in LM from ducts from WT mice. This possibility was excluded by the finding that 50 μM HOE 694 inhibited more than 85% of luminal Na⁺-dependent H⁺ efflux in ducts from *NHE2*^{-/-} mice. These findings also suggest that sensitivity to HOE 694 cannot be used in all cases to discern the NHE isoform expressed in a given tissue or cell type.

Another alternative explanation for the lack of effect of deletion of *NHE2* is that the NHE2 expressed in the LM of the pancreatic duct is not active or may not function as a Na⁺/H⁺ exchanger. Some support for this unconventional interpretation comes from the absence of any apparent abnormalities in renal, intestinal, or

pancreatic function in the *NHE2*^{-/-} animals (ref. 15 and this study). In addition, deletion of *NHE2* had no effect on Na⁺-dependent H⁺ efflux in the proximal tubule (21). Hence, the role of luminal NHE2 in epithelial physiology remains to be found.

In contrast to the results with *NHE2*, deletion of the *NHE3* gene reduced luminal pancreatic ductal Na⁺-dependent H⁺ efflux by 45%. The unexpected finding in this case was the 85–90% inhibition of overall luminal H⁺ transport by 50 μM HOE 694. This indicates that most of the NHE3-mediated activity in the pancreatic duct is inhibited by 50 μM HOE 694. Yet, 50 μM HOE 694 had no effect on Na⁺-dependent H⁺ transport in the LM of the mouse proximal tubule (21). It is therefore conceivable that the pancreatic duct expresses an NHE3 splice variant different from that expressed in the kidney. Further cloning efforts are needed to examine this possibility. Alternatively, posttranslational modification of NHE3 may confer tissue-specific sensitivity to HOE 694 (and possibly other amiloride analogues). This raises further questions regarding the general use of sensitivity to amiloride and its analogues as diagnostic tools of NHE isoforms.

An additional unexpected finding was the novel HOE 694-inhibitable; Na⁺-dependent H⁺ efflux mechanism in the LM of pancreatic duct. Recently, we reported analogous activity in the proximal tubule, with the difference that the renal activity required 100 μM EIPA for maximal inhibition (21). The particular significance of this activity is that it mediates as much as 55% of luminal Na⁺-dependent H⁺ efflux. Hence, it may have an important physiological role in HCO₃⁻ absorption in various tissues.

At present, we do not know what protein may mediate this activity. An obvious possibility is an as-yet unknown NHE isoform, different from NHE1–5. Additional possibilities are the newly described Na⁺ and HCO₃⁻ cotransporter NBC3 (24) or a similar isoform. Unique features of NBC3, when compared with other NBC isoforms, are that it can transport HCO₃⁻ and OH⁻, it is inhibitable by amiloride analogues, and it is insensitive to the stilbene derivative DIDS (24). These features are reminiscent of those found for the novel luminal Na⁺-dependent H⁺ (or HCO₃⁻) transport in the pancreatic duct (this study) and proximal tubule (21). We are in the process of evaluating the contribution of this or a similar transporter to luminal Na⁺-dependent H⁺ efflux.

Table 2
Buffer capacity of mouse pancreatic ducts at pH_i 7.0

	Resting (ΔmM NH ₄ ⁺ /ΔpH _i)	Forskolin (5 μM)
WT	47.4 ± 5.4 (n = 5)	49.0 ± 5.5 (n = 5)
<i>NHE2</i> ^{-/-}	45.9 ± 2.7 (n = 4)	47.1 ± 4.1 (n = 4)
<i>NHE3</i> ^{-/-}	45.4 ± 8.7 (n = 4)	42.8 ± 5.6 (n = 4)

In each experiment, the buffer capacity (β_i) showed a negative linear relationship with pH_i between 6.3 and 7.3. The β_i at pH_i = 7.0 was calculated from the correlative equation.

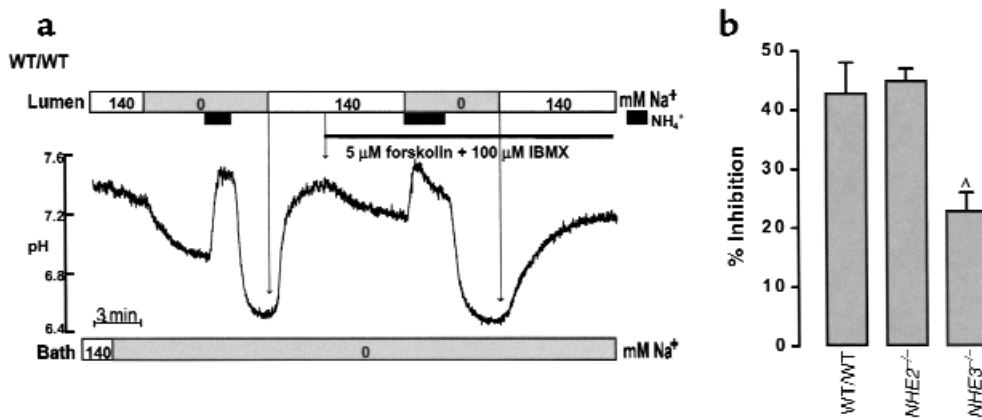


Figure 6

Regulation of luminal NHE activity by cAMP. After the control period, ducts were treated with 5 μM forskolin and 100 μM IBMX for 5 minutes. (a) A representative experiment using the pancreatic duct of a WT mouse. (b) Summary of the multiple experiments. Forskolin treatment reduced luminal NHE activity by $43 \pm 5\%$ ($n = 5$) in WT mice, $45 \pm 2\%$ ($n = 6$) in *NHE2*^{-/-} mice, and $23 \pm 6\%$ ($n = 6$) in *NHE3*^{-/-} mice. $^*P < 0.05$ compared with WT.

The pancreatic duct secretes the bulk of HCO_3^- found in secretin-stimulated pancreatic juice. Secretin effects in duct cells are mediated by stimulation of adenylyl cyclase. It is believed that the increase in cAMP activates the cystic fibrosis transmembrane conductance regulator (CFTR) to facilitate HCO_3^- secretion by a $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism. Na^+ -dependent H^+ secretion was not expected to be present in the LM of pancreatic ducts, because it can reduce pancreatic HCO_3^- and fluid secretion by secreting H^+ and absorbing Na^+ (and consequently water) from the luminal fluid. Therefore, it was of particular interest to determine possible regulation of the luminal NHE transporters by cAMP. Pancreatic NHE3 and the luminal Na^+ -dependent H^+ efflux transporter are inhibited by a cAMP-dependent mechanism. Thus, in pancreatic ducts from both WT and *NHE2*^{-/-} animals, an increase in cAMP reduced NHE activity by an average of about 45%. Stimulation with cAMP reduced the novel Na^+ -dependent H^+ efflux by about 25%, which amounts to about 12.5% of total luminal

Na^+ -dependent H^+ efflux. Thus, cAMP stimulation inhibited NHE3 activity by about 37.5%. Regulatory inhibition of NHE3 by cAMP in the kidney proximal tubule is well understood (25). Protein kinase-mediated (PKA-mediated) phosphorylation may regulate NHE3 activity directly or through changes in the availability of NHERF (a PDZ motif containing protein) for interaction with NHE3 (26). In this respect, it is interesting that NHERF can also downregulate the activity of NBC transporters (27). Such a mechanism may be related to the inhibition of the novel Na^+ -dependent H^+ efflux by cAMP stimulation.

What can the physiological relevance of luminal Na^+ -dependent H^+ efflux in the HCO_3^- secreting pancreatic duct be? A plausible scenario is illustrated in the models shown in Figure 7. The main pancreatic duct maintains a resting transepithelial potential difference of -5 mV, lumen negative (28), and has Na^+ -leaky tight junctions (1). The duct also expresses CFTR and has $\text{Cl}^-/\text{HCO}_3^-$ exchange activity that is modulated by the PKA-phos-

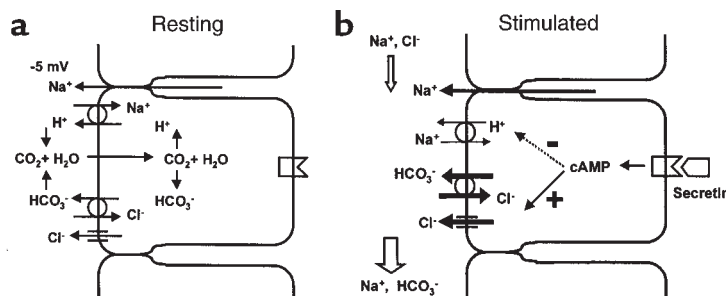


Figure 7

Roles of luminal Na^+ -dependent H^+ efflux mechanisms in pancreatic duct. (a) The resting pancreatic duct maintains a transepithelial voltage of -5 mV, lumen negative, and has a leaky tight junction. Hence, Na^+ flows from the interstitial space to the luminal space. The luminal Na^+ -dependent H^+ efflux mechanisms absorb the Na^+ in exchange for H^+ . H^+ efflux is equivalent to HCO_3^- reabsorption, which is secreted by the basal $\text{Cl}^-/\text{HCO}_3^-$ exchange activity. (b) When feeding, the gastrointestinal hormone secretin is released from intestinal cells to stimulate pancreatic secretion by increasing intracellular cAMP in duct cells. The activity of the luminal Na^+ -dependent H^+ efflux mechanisms are downregulated by a cAMP-dependent mechanism to reduce Na^+ - HCO_3^- reabsorption.

phorylated CFTR (18). The basal $\text{Cl}^-/\text{HCO}_3^-$ exchange activity of the pancreatic duct is about 50% of the cAMP-stimulated activity (18). Basal $\text{Cl}^-/\text{HCO}_3^-$ exchange and Na^+ leaking through the tight junction will result in a basal secretion of a fluid rich in Na^+ and HCO_3^- . To prevent loss of Na^+ and HCO_3^- under the basal state, these ions must be reabsorbed from the luminal fluid. The Na^+ -dependent H^+ efflux mechanisms described here can perform this function in a mode analogous to that used by the proximal tubule to absorb Na^+ and HCO_3^- (Figure 7a). Na^+ and HCO_3^- absorption will also increase absorption of fluid from the duct lumen. Low pancreatic secretion in the interdigestive basal state is of particular importance in intermittent feeders such as humans (22). There is evidence for Na^+ and HCO_3^- absorption by the duct in the basal state. In fact, basal secretion of digestive enzymes by acinar cells is about 10% of maximal. By contrast, basal secretion of HCO_3^- by the duct is only 1–2% of maximal. Equally important, the basal secretory fluid contains high levels of Cl^- (22). This suggests that indeed, the pancreatic duct absorbs HCO_3^- rather than Cl^- under basal conditions.

When feeding, the gastrointestinal hormone secretin is released from intestinal cells to stimulate pancreatic ductal fluid and HCO_3^- secretion by increasing cellular cAMP (1). cAMP activates CFTR (29), which in turn results in activation of $\text{Cl}^-/\text{HCO}_3^-$ exchange (18, 30). Consequently, a large amount of HCO_3^- is secreted into the lumen, which is followed by Na^+ and water transport through the leaky tight junctions. The results presented here show that in parallel with stimulation of HCO_3^- secretion, cAMP inhibits Na^+ -dependent H^+ secretion by NHE3 and the novel luminal efflux mechanism. This will reduce Na^+ and HCO_3^- absorption to allow $\text{Cl}^-/\text{HCO}_3^-$ exchange and CFTR to produce HCO_3^- -rich pancreatic juice.

In summary, we believe that this study highlights an underappreciated but important function of the pancreatic duct: the control of pancreatic secretion and the composition of the pancreatic juice under basal conditions. It appears that a luminal NHE3 mechanism and a novel Na^+ -dependent, amiloride-sensitive H^+ efflux mechanism reabsorb Na^+ , HCO_3^- , and fluid, probably to produce an acidic, Cl^- -rich pancreatic juice under resting conditions. Upon cell stimulation by agonists that act through cAMP, these mechanisms are inhibited to allow production of an HCO_3^- -rich pancreatic juice.

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