Novel amiloride-sensitive sodium-dependent proton secretion in the mouse proximal convoluted tubule

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The proximal convoluted tubule (PCT) reabsorbs most of the filtered bicarbonate. Proton secretion is believed to be mediated predominantly by an apical membrane Na^+/H^+ exchanger (NHE). Several NHE isoforms have been cloned, but only NHE3 and NHE2 are known to be present on the apical membrane of the PCT. Here we examined apical membrane PCT sodium-dependent proton secretion of wild-type (*NHE3*$^{+/+}$/*NHE2*$^{+/+}$), *NHE3*$^{-/-}$, *NHE2*$^{-/-}$, and double-knockout *NHE3*$^{-/-}$/*NHE2*$^{-/-}$ mice to determine their relative contribution to luminal proton secretion. *NHE2*$^{-/-}$ and wild-type mice had comparable rates of sodium-dependent proton secretion. Sodium-dependent proton secretion in *NHE3*$^{-/-}$ mice was approximately 50% that of wild-type mice. The residual sodium-dependent proton secretion was inhibited by 100 μM 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA). Luminal sodium-dependent proton secretion was the same in *NHE3*$^{-/-}$/*NHE2*$^{-/-}$ as in *NHE3*$^{-/-}$ mice. These data point to a previously unrecognized Na^+-dependent EIPA-sensitive proton secretory mechanism in the proximal tubule that may play an important role in acid-base homeostasis.

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Introduction

The proximal tubule reabsorbs 80% of the filtered bicarbonate and 60% of the filtered chloride. Based on Na^+-dependence and amiloride sensitivity, the Na^+/H^+ antiporter is assumed to mediate approximately 70% of proximal tubule luminal proton secretion for bicarbonate reabsorption (1, 2). In addition, the Na^+/H^+ antiporter in parallel with a Cl^-/base exchanger is believed to be responsible for active transcellular NaCl transport in this segment (3, 4).

A number of isoforms of the Na^+/H^+ exchanger have been cloned (5–9). Three isoforms of the Na^+/H^+ exchanger have been localized to the proximal tubule. NHE1 has a wide distribution in mammalian tissues and is localized to the basolateral membrane (10). NHE3 and NHE2 have been localized to the apical membrane of the proximal tubule (12–15). Nonetheless, recent studies have suggested that NHE3 is the sole isoform mediating Na^+/H^+ exchange activity on the apical membrane of the proximal convoluted tubule (16, 17).

Recently, mice lacking NHE3 and NHE2 have been generated (18, 19). In the present in vitro microperfusion study, we perfused proximal convoluted tubules from wild-type mice and mice with targeted disruption of NHE3 and NHE2 as well as a double knockout to examine the relative contributions of NHE3 and NHE2 to sodium-dependent proton secretion in this nephron segment. Our data indicate that NHE3, but not NHE2, contributes to luminal proton secretion in the proximal convoluted tubule. NHE3$^{-/-}$ mice and NHE3$^{-/-}$/NHE2$^{-/-}$ have only a 50% reduction in EIPA-sensitive sodium-dependent proton secretion. Thus, as much as 50% of amiloride-sensitive, sodium-dependent proton secretion may be mediated by a previously unrecognized novel mechanism in the proximal tubule of these mice.

Methods

Knockout mice. Mice with targeted disruption of NHE2 and NHE3 were generated as described previously (18, 19). We have previously demonstrated that NHE2$^{-/-}$ and NHE3$^{-/-}$ mice have a distinct phenotype (18, 19). The mutant mRNA for NHE3 lacks codons for amino acids 320–831, which encodes sequences required for Na^+/H^+ exchange (18), and the NHE2$^{-/-}$ has an aberrant splice that deletes 241 nucleotides and causes a frameshift in the coding

sequence, resulting in the elimination of 641 of 813 amino acids (19). We have previously shown that NHE3$^{-/-}$ mice do not express NHE3 (18, 20). Likewise, it has been demonstrated previously that NHE2$^{-/-}$ mice do not express NHE2 (19, 21). Homozygous NHE2$^{-/-}$, NHE3$^{-/-}$, NHE3$^{-/-}$/NHE2$^{-/-}$, and wild-type (NHE3$^{+/+}$/NHE2$^{+/+}$) mice were generated by mating of heterozygotes. All animals were genotyped using tail DNA before study. Animals were allowed free access to food and water and were studied at 1–2 months of age. Animals were sacrificed by cervical dislocation after ether anesthesia.

In vitro microperfusion. Isolated segments of proximal convoluted tubules were perfused using concentric glass pipettes as described previously (4, 22). Briefly, tubules were dissected in Hanks' balanced salt solution containing 137 mm NaCl, 5 mm KCl, 0.8 mm $MgSO_4$, 0.33 mm Na_2HPO_4, 0.44 mm KH_2PO_4, 1 mm $MgCl_2$, 10 mm Tris (hydroxymethyl) aminomethane hydrochloride, 0.25 mm $CaCl_2$, and the metabolic substrates 2 glutamine, 2 heptanoic acid, and 2 lactate at 4°C. Tubules were transferred to a 0.2-mL chamber in which the bathing solution was preheated to 38°C.

Measurement of pH_I. The fluorescent dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was used to measure pH_i as described previously (4, 22–24). pH_i was measured using a Nikon inverted epifluorescent microscope (Nikon, Japan) attached to a PTI Ratiomaster (Photon Technology International, South Brunswick, New Jersey, USA) at a rate of 30 measurements per second. A variable diaphragm was placed over the area to be measured. To calculate pH from the ratio of fluorescence (F_{500}/F_{450}), a nigericin calibration curve was performed as described previously (4, 22, 23).

The solutions used to perfuse and bath the tubules are listed in Table 1. dpH_i/dt was measured from the slope of the change in pH_i immediately after a luminal fluid change as described in detail previously (22, 24).

Table 1
Solutions

	Bath A	Luminal Na^+ B	Luminal ONa^+ C
NaCl	140	115	–
$NaHCO_3$	5	25	–
NMDG-Cl	–	–	115
Choline HCO_3	–	–	25
KCl	5	–	–
K_2HPO_4	–	2.5	2.5
$MgCl_2$	–	1	1
$MgSO_4$	1	–	–
Na_2HPO_4	1	–	–
Glucose	5	–	–
L-Alanine	5	–	–
Urea	5	–	–
$CaCl_2$	1.8	1.8	1.8
Heptanoic Acid	2	–	–
pH	6.6	7.4	7.4

All constituents are in mM. All solutions were adjusted to an osmolality of 295 mOsm/kg H_2O.

SDS-PAGE and immunoblotting. Kidneys were rapidly removed and placed in an ice-cold isolation buffer containing 300 mM mannitol, 16 mM HEPES, 5 mM EGTA, titrated to pH 7.4 with Tris, aprotinin (2 μg/mL), leupeptin (2 μg/mL) and phenylmethylsulfonyl fluoride (100 μg/mL). The cortex was dissected and then homogenized with 20 strokes with a Teflon-glass homogenizer at 4°C. Brush border membrane vesicles (BBMV) were then isolated by differential centrifugation and magnesium precipitation as described previously (25). The final BBMV fraction was resuspended in isolation buffer. Protein was assayed using the bicinchoninic acid assay (BCA Protein Assay; Pierce Chemical Co., Rockford, Illinois, USA) using BSA as the standard.

BBMV protein (50 μg/lane) was denatured and separated on a 7.5% polyacrylamide gel as described previously (25). The proteins were transferred to polyvinylidene difluoride membrane overnight at 140 mA at 4°C. The blot was blocked with fresh Blotto (5% nonfat milk, 0.05% Tween 20, and PBS [pH 7.4]) for 1 hour, and then a primary antibody to rat NHE3 (gift from O. Moe, University of Texas Southwestern Medical Center, Dallas, Texas, USA) or rat NHE2 (Chemicon International Inc., Temecula, California, USA) was added at a 1:1000 dilution and incubated for 12 hours at 4°C. β-Actin antibody (Sigma Chemical Co., St. Louis, Missouri, USA) was added at a 1:10,000 dilution. The blot was washed with PBS containing 1% Tween, and then the secondary horseradish peroxidase–conjugated anti-rabbit immunoglobulin for NHE2 and NHE3 and anti-mouse immunoglobulin for β-actin was added for 1 hour at 1/10,000 dilution in Blotto at room temperature. The blot was washed with PBS containing 1% Tween, and enhanced chemiluminescence was used to detect bound antibody (Amersham Life Sciences, Inc., Arlington Heights, Illinois, USA).

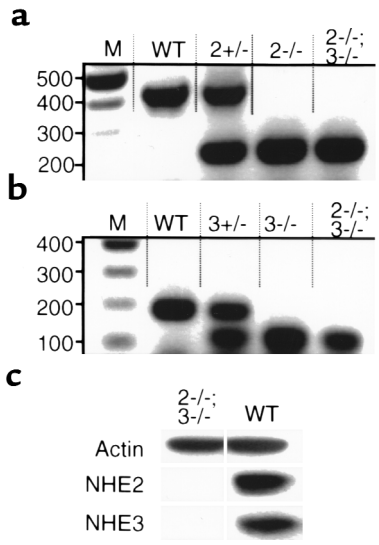
Statistics. Data are expressed as means ± SE. ANOVA and the Student's *t* test for unpaired data were used to determine statistical significance.

Results

Knockout mice. As shown in Figure 1, NHE2$^{-/-}$, NHE3$^{-/-}$, and NHE3$^{-/-}$/NHE2$^{-/-}$ mice had deletion of these respective isoforms of the Na^+/H^+ exchanger. Figure 1 also shows a Western blot demonstrating that NHE2 and NHE3 were present in renal brush-border membrane vesicles from wild-type mice but totally absent from the NHE3$^{-/-}$/NHE2$^{-/-}$ mice.

Effect of 100 μM EIPA and 50 μM HOE694 on Na^+/H^+ antiporter activity in C57Bl/6 Mice. Na^+/H^+ antiporter activity was measured using the same assay as described previously in adult rat proximal convoluted tubules perfused in vivo and rabbit proximal convoluted tubules perfused in vitro (22, 24). Mice proximal convoluted tubules (PCT) were perfused with an ultrafiltrate-like solution without glucose and amino acids (Table 1, solution B). Organic solutes were

Figure 1
(**a**) PCR analysis of tail DNA from wild-type, NHE2$^{-/+}$, NHE2$^{-/-}$, and NHE3$^{-/-}$/NHE2$^{-/-}$ mice. Primers were designed to amplify a 450-bp product from wild-type NHE2 sequence, or a 221-bp product from the mutant allele that included part of the inserted neomycin resistance gene. (**b**) PCR analysis of tail DNA from wild-type, NHE3$^{+/-}$, NHE3$^{-/-}$, and NHE3$^{-/-}$/NHE2$^{-/-}$ mice. As is shown, primers amplified a 199-bp product of wild-type NHE3 or a 113-bp product of the mutant allele that contained part of the inserted neomycin resistance gene. (**c**) Western blot of renal brush border membranes from wild-type and NHE3$^{-/-}$/NHE2$^{-/-}$ mice. Membranes were probed with anti-rat NHE2 and anti-rat NHE3 antibodies that both detected approximately 90-kDa proteins in brush-border membranes from wild-type mice (WT). Note the total absence of NHE2 and NHE3 protein from renal brush-border membranes in NHE3$^{-/-}$/NHE2$^{-/-}$ mice. β-Actin shows identical loading of the immunoblots.



omitted from the luminal solution because sodium-coupled glucose and amino acid transport depolarizes the basolateral membrane, which may affect bicarbonate exit, an electrogenic process (23). 4-Acetamido-4′-isothiocyano-stilbene-2,2′ disulfonic acid (1 mM SITS) was present in the bathing solution to inhibit the sodium bicarbonate cotransporter, a major regulator of intracellular pH_i in proximal convoluted tubules (22, 24). The bathing solution (Table 1, solution A) had a bicarbonate concentration of 5 mM and a pH of 6.6 to compensate for the cell alkalinization caused by the addition of bath SITS (22, 24). Under these conditions, the rate of change in pH_i in response to a change in luminal sodium concentration has been shown to be a measure of Na^+/H^+ antiporter activity (22–24). In the experimental period, luminal sodium was removed (Table 1, Solution C) and dpH_i/dt measured.

The effect of luminal sodium removal on dpH_i/dt is shown in Figure 2. The rate of sodium-dependent proton secretion in wild-type mice was comparable to that measured with these solutions in rat proximal convoluted tubules in vivo (24) and in rabbit proximal convoluted tubules perfused in vitro (22). We next examined the effect of 50 μM (3-methylsulfonyl-4-piperidinobenzoyl) guanidine methanesulfonate (HOE694) on apical membrane Na^+/H^+ antiporter activity. As shown in Figure 2, there was no effect of luminal HOE694 on sodium-dependent proton secretion. This dose of HOE694 is expected to reduce NHE2 activity by 90% (26). Luminal 100 μM 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) reduced apical sodium-dependent proton secretion by approximately 80% ($P < 0.001$). These data are consistent with NHE2 not playing a significant role in proximal convoluted tubule acidification.

Na^+/H^+ antiporter activity in NHE3$^{+/+}$/NHE2$^{+/+}$(wild type), NHE3$^{-/-}$, NHE2$^{-/-}$, and NHE3$^{-/-}$/NHE2$^{-/-}$ (double knockout) proximal convoluted tubules. In the next series of experiments, we measured sodium-dependent proton secretion in NHE3$^{+/+}$/NHE2$^{+/+}$(wild type), NHE3$^{-/-}$, NHE2$^{-/-}$, and NHE3$^{-/-}$/NHE2$^{-/-}$ mice. Typical tracings showing the effect of luminal sodium removal on pH_i are shown in Figure 3. dpH_i/dt in these experiments are shown in Figure 4. Sodium-dependent proton secretion in wild-type mice was comparable to that measured in C57B1/6 mice and was inhibited by luminal 100 μM EIPA. The rate of sodium-dependent proton secretion in NHE2$^{-/-}$ mice was comparable to that of wild-type mice. The rate of sodium-dependent proton secretion was significantly lower in NHE3$^{-/-}$ mice than in wild-type mice. However, there was only a 50% reduction in sodium-dependent proton secretion compared with control mice. Most of the residual sodium-dependent proton secretion in NHE3$^{-/-}$ mice was inhibited by luminal 100 μM EIPA.

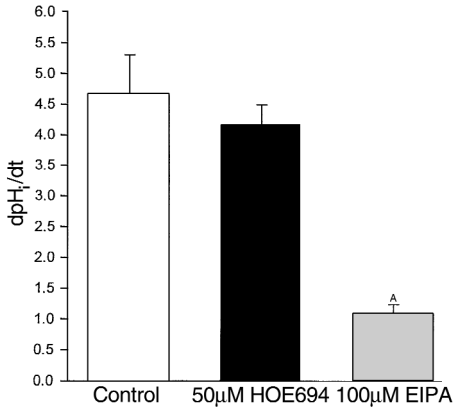


Figure 2
Proximal convoluted tubule sodium-dependent proton secretion in C57Bl/6 mice. Activity was assayed as the rate of change in intracellular pH_i upon luminal sodium removal. Sodium-dependent proton secretion was inhibited by addition of luminal 100 μM EIPA but not 50 μM HOE694. $^AP < 0.001$ vs. control and HOE groups.

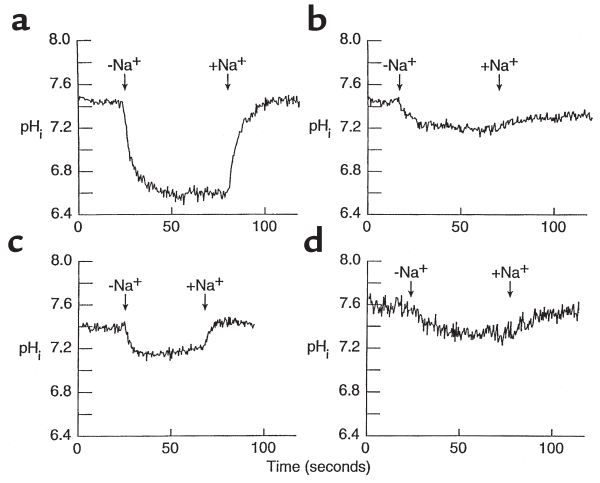


Figure 3
Typical tracings showing the effect of luminal sodium removal and addition (arrows) on pH_i. (**a** and **b**) PCT from NHE3$^{+/+}$/NHE2$^{+/+}$(wild type) in the absence and presence of luminal 100 μM EIPA, respectively. (**c**) PCT from a NHE3$^{-/-}$/NHE2$^{-/-}$ mouse. (**d**) NHE3$^{-/-}$/NHE2$^{-/-}$ proximal tubule with luminal 100 μM EIPA.

We examined whether upregulation of NHE2 was responsible for the EIPA-sensitive sodium-dependent proton secretion in NHE3$^{-/-}$ mice. As shown in Figure 5, the rate of proximal convoluted tubule sodium-dependent proton secretion was the same in NHE3$^{-/-}$ mice in the presence and absence of HOE694. In addition, as shown in Figure 4, the rate of sodium-dependent proton secretion measured in tubules from NHE3$^{-/-}$/NHE2$^{-/-}$ double-knockout mice was not significantly different than that from NHE3$^{-/-}$ mice.

Discussion

Proximal tubule acidification is believed to be mediated predominantly by a luminal Na^+/H^+ antiporter (1, 2). In the present in vitro microperfusion study, we used NHE3$^{-/-}$, NHE2$^{-/-}$, and NHE3$^{-/-}$/NHE2$^{-/-}$ mice to examine whether there was additional sodium-dependent proton secretion in this segment. We found that there was EIPA-sensitive, sodium-dependent proton secretion in both NHE3$^{-/-}$ mice and NHE3$^{-/-}$/NHE2$^{-/-}$ mice consistent with expression of a previously unrecognized proton secretory mechanism in this segment.

Mice lacking NHE3 thrive, but have a lower blood pressure, higher serum aldosterone level, and higher renal renin mRNA expression than do wild-type mice (18). These findings are consistent with NHE3$^{-/-}$ mice being relatively volume contracted compared with the wild-type mice. Despite diarrhea and a reduced rate of proximal tubule bicarbonate reabsorption, NHE3$^{-/-}$ mice have only a slightly lower serum pH (7.27 ± 0.02 versus 7.33 ± 0.01; $P < 0.02$) and serum bicarbonate concentration (21.1 ± 0.9 versus 24.2 ± 0.6 mM; $P < 0.02$) than do wild-type mice (18). NHE3$^{-/-}$ mice had a greater intestinal absorptive area, distal colonic short-circuit current, colonic H^+, K^+-ATPase, and distal colon β- and γ-ENaC mRNA expression, as well as renal Cl^-/HCO_3^- exchange (AE1) and renal α-ENaC mRNA abundance, than do wild-type mice (18). Recently, a compensatory increase in the rate of bicarbonate absorption was demonstrated in the cortical collecting duct and outer medullary collecting duct of NHE3$^{-/-}$ mice (27). This study was consistent with an increase in H^+-ATPase activity in the cortical collecting duct and an increase in both H^+-ATPase and H^+-K^+-ATPase activity in the medullary collecting duct. Collectively, these studies demonstrate that mice lacking NHE3 have compensatory means for intestinal and renal salt conservation and renal acidification.

Mice with targeted disruption of NHE2 have a distinctly different phenotype than NHE3$^{-/-}$ mice (19). NHE2$^{-/-}$ mice do not have a metabolic acidosis, and their plasma aldosterone concentration was comparable to that of wild-type mice. NHE2$^{-/-}$ mice had reduced viability of gastric parietal cells and decreased net gastric acid secretion. The fact that the serum pH, electrolyte composition, and plasma aldosterone con-

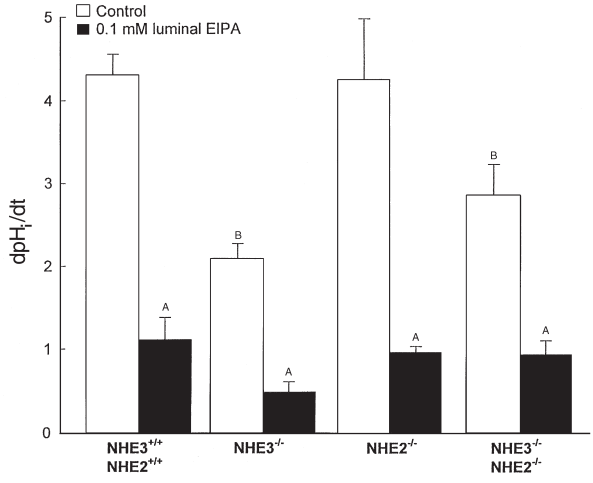
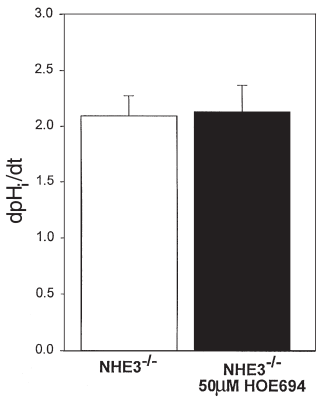


Figure 4
PCT sodium-dependent proton secretion in NHE3$^{+/+}$/NHE2$^{+/+}$ (wild type), NHE3$^{-/-}$, NHE2$^{-/-}$, and NHE3$^{-/-}$/NHE2$^{-/-}$ mice. Activity was comparable in NHE3$^{+/+}$/NHE2$^{+/+}$(wild type) and NHE2$^{-/-}$ mice. Sodium-dependent proton secretion was significantly less in NHE3$^{-/-}$ and NHE3$^{-/-}$/NHE2$^{-/-}$ mice than in wild-type mice. In all groups, luminal 100 μM EIPA inhibited sodium-dependent proton secretion. $^AP < 0.01$ vs. respective control value. $^BP < 0.05$ vs. control value of wild-type and NHE2$^{-/-}$ mice.

Figure 5
PCT sodium-dependent proton secretion in NHE3$^{-/-}$ mice in the presence and absence of luminal 50 μM HOE694.



centration of NHE2$^{-/-}$ mice were comparable to those of wild-type mice suggest that these mice do not have a significant renal acidification defect and that they are not volume depleted. Unfortunately, we were unable to characterize further the NHE3$^{-/-}$/NHE2$^{-/-}$ mice. NHE3$^{-/-}$/NHE2$^{-/-}$ mice were generated by mating NHE3$^{-/+}$/NHE2$^{-/+}$ mice. Only a few double-knockout mice thrived and survived for this study.

Both NHE3 and NHE2 are present on the apical membrane of the proximal tubule. The relative sensitivities of these isoforms to amiloride and its analogues have been used to predict the relative contribution of NHE3 and NHE2 to proximal tubule proton secretion using renal brush-border membrane vesicles (17, 26). Rat brush-border Na^+/H^+ antiporter activity had similar IC_{50} values for amiloride, EIPA, HOE694, and 5-*N*-dimethyl amiloride as that found previously for NHE3 (17, 26). Renal brush-border membrane Na^+/H^+ exchange activity was minimally affected by HOE694, at a dose that should have totally inhibited NHE2 (17). These data are consistent with NHE2 not playing a role in mediating Na^+/H^+ antiporter activity in the proximal tubule. Our data confirm these observations. The rate of sodium-dependent proton secretion in NHE2$^{-/-}$ mice was comparable to that of wild-type mice.

A previous study has used wild-type (NHE3$^{+/+}$) and NHE3$^{-/-}$ mice to examine the contribution of NHE3 in mediating proximal tubule bicarbonate absorption using in vivo microperfusion (16). Addition of 100 μM EIPA to the luminal perfusate of NHE3$^{+/+}$ mice resulted in a 40% reduction in the rate of bicarbonate reabsorption. Luminal 1 μM Bafilomycin, an inhibitor of the vacuolar H^+-ATPase, inhibited the rate of bicarbonate reabsorption by 22% in NHE3$^{+/+}$ mice. In NHE3$^{-/-}$ mice, the rate of bicarbonate absorption was 51.2 ± 3.4 compared with 110 ± 9.6 pmol/min per millimeter in NHE3$^{+/+}$ mice. Luminal 100 μM EIPA resulted in a 7% reduction in the rate of bicarbonate absorption in NHE3$^{-/-}$ mice, which was not statistically different from that of control. The authors concluded that proximal tubule acidification is not mediated by NHE2 or any other EIPA-sensitive NHE isoform (16), a conclusion at variance with this study. However, 38% of bicarbonate transport in NHE3$^{+/+}$ mice in this study were not inhibited by either 100 μM luminal EIPA or 1 μM luminal Bafilomycin (16).

The difference in technique used to study the Na^+/H^+ antiporter activity using in vitro microperfusion and in vivo microperfusion provides a likely explanation for the difference in results. In vivo microperfusion bicarbonate flux studies use slow perfusion rates (~20 nL/min) and relatively long tubule segments (~1.5 mm) to assay the rate of volume and bicarbonate reabsorption. The luminal perfusate initially contained 100 μM EIPA in both studies. However, a previous in vivo rat microperfusion study showed that the amiloride analogue t-butyl amiloride is almost totally reabsorbed along a perfused proximal convoluted tubule (1). Thus, the proximal tubule is permeable to lipophilic amiloride analogues, and it is likely that the mean luminal concentration of EIPA, another lipophilic amiloride analogue, was significantly less than the 100 μM in the initial luminal perfusate. In the current study, tubules were perfused at a rate approximately 100-fold faster than those used in vivo, and Na^+/H^+ antiporter activity was measured in the proximal tubule cells immediately beyond the perfusion pipette. However, what was measured in these studies was sodium-dependent proton secretion under artificial, nonphysiologic conditions. Thus, the relative contribution of this novel amiloride-sensitive luminal proton secretory mechanism to proximal tubule bicarbonate absorption under physiologic conditions in vivo is unknown.

In summary, the present study compared the rate of sodium-dependent proton secretion in NHE3$^{-/-}$ mice and NHE2$^{-/-}$ mice to that of wild-type mice. Our data show that targeted disruption of NHE3 reduced sodium-dependent proton secretion by only 50%, whereas sodium-dependent proton secretion in NHE2$^{-/-}$ mice was comparable to control mice. NHE3$^{-/-}$ and NHE3$^{-/-}$/NHE2$^{-/-}$ mice have residual apical EIPA-sensitive sodium-dependent proton secretion consistent with either another Na^+/H^+ isoform, an apical sodium-dependent base cotransporter, or a novel sodium-dependent proton secretory mechanism. Of note, these studies were performed in the presence of luminal bicarbonate. Recently, an EIPA-sensitive member of the sodium bicarbonate cotransporter family that can function in the absence of CO_2 and bicarbonate has been described (28). Elucidation of the mechanism for the luminal sodium-dependent proton secretion in these NHE3$^{-/-}$ mice will clearly require additional studies. However, independent of the exact mechanism responsible for this novel sodium-dependent proton secretion, the finding that it contributes to as much as 50% of sodium-dependent proton secretion in the proximal convoluted tubule of NHE3$^{-/-}$ mice suggests that it may play an important role renal acidification.

Acknowledgments

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