NHE2 is the main apical NHE in mouse colonic crypts but an alternative Na\(^+\)-dependent acid extrusion mechanism is upregulated in NHE2-null mice

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Guan, Yanfang, Jin Dong, Lixuan Tackett, Jamie W. Meyer, Gary E. Shull, and Marshall H. Montrose. NHE2 is the main apical NHE in mouse colonic crypts but an alternative Na\(^+\)-dependent acid extrusion mechanism is upregulated in NHE2-null mice. Am J Physiol Gastrointest Liver Physiol 291: G689–G699, 2006. First published May 11, 2006; doi:10.1152/ajpgi.00342.2005.—The mechanism of apical Na\(^+\)-dependent H\(^+\) extrusion in colonic crypts is controversial. With the use of confocal microscopy of the living mouse distal colon loaded with BCECF or SNARF-5F (fluorescent pH sensors), measurements of intracellular pH (pH\(_i\)) in epithelial cells at either the crypt base or colonic surface were reported. After cellular acidification, the addition of luminal Na\(^+\) stimulated similar rates of pH\(_i\) recovery in cells at the base of distal colonic crypts of wild-type or Na\(^+/\)H\(^+\) exchanger isoform 2 (NHE2)-null mice. In wild-type crypts, 20 \(\mu\)M HOE694 (NHE2 inhibitor) blocked 68–75% of the pH\(_i\) recovery rate, whereas NHE2-null crypts were insensitive to HOE694, the NHE3-specific inhibitor S-1611 (20 \(\mu\)M), or the bicarbonate transport inhibitor 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS; 1 mM). A general NHE inhibitor, 5-(N-ethyl-N-isopropyl)amiloride (EIPA; 20 \(\mu\)M), inhibited pH\(_i\) recovery in NHE2-null mice (46%) but less strongly than in wild-type mice (74%), suggesting both EIPA-sensitive and -insensitive compensatory mechanisms. Transepithelial Na\(^+\) leakage followed by activation of basolateral NHE1 could confound the outcomes; however, the rates of Na\(^+\)-dependent pH\(_i\) recovery were independent of transepithelial leakiness to lucifer yellow and were unchanged in NHE1-null mice. NHE2 was immuno-localized on apical membranes of wild-type crypts but not NHE2-null tissue. NHE3 immunoreactivity was near the colonic surface but not at the crypt base in NHE2-null mice. Colonic surface cells from wild-type mice demonstrated S1611- and HOE694-sensitive pH\(_i\) recovery in response to luminal sodium, confirming a functional role for both NHE3 and NHE2 at this site. We conclude that constitutive absence of NHE2 results in a compensatory increase in a Na\(^+\)-dependent, EIPA-sensitive acid extruder distinct from NHE1, NHE3, or SITS-sensitive transporters.

Slt9a2; Slt9a3; BCECF; SNARF-5F; intracellular pH; laser scanning confocal microscopy; colon; Na\(^+/\)H\(^+\) exchanger

IN THE MAMMALIAN COLON, Na\(^+\)/H\(^+\) exchange across apical membranes plays an essential role in fluid and Na\(^+\) absorption as well as in absorption stimulated by short-chain fatty acids (18, 21, 24, 25). Among the nine known Na\(^+/\)H\(^+\) exchanger (NHE; gene locus symbol, Slt9aX) isoforms, NHE1–NHE3 occur in the colon (for a review, see Ref. 28). A potentially unique Cl\(^–\)-dependent NHE has been reported in rat and rabbit crypt cells (14), but this protein has not been observed in mouse colonic crypts (2, 9). NHE1 (Slt9a1) is exclusively present in the colonocyte basolateral membrane (6) and has a major role in intracellular pH (pH\(_i\)) homeostasis (27). NHE2 (Slt9a2) and NHE3 (Slt9a3) are expressed in apical membranes in the colon (6, 7, 16) and are therefore the only identified isoforms that could serve absorptive functions in this tissue.

The role of NHE3 in intestinal and/or colonic Na\(^+\) absorption has been clearly demonstrated by the occurrence of diarrhea in NHE3-null mice (25). The absence of a diarrhea phenotype or any apparent Na\(^+\)-absorptive defect in NHE2-null mice was taken as evidence that NHE2 does not play an important role in Na\(^+\) absorption (13, 24). In addition, diarrhea in NHE3-null mice was not further aggravated in NHE2/NHE3 double-knockout mice (19), as would have been expected if NHE2 provided a major absorptive capacity. However, rat colonic NHE2 and NHE3 are similarly increased by Na\(^+\) depletion (17). On the basis of inhibitor sensitivity, NHE2 but not NHE3 appeared to be responsible for the enhanced Na\(^+\) absorption in response to a low-Na\(^+\) diet in the chicken colon (11). Pharmacological evidence also suggests that NHE2 rather than NHE3 may be important for basal Na\(^+\) absorption in the rat colon (8). Thus, the function of NHE2 in the colon remains uncertain.

NHE2 and NHE3 mRNA and protein are expressed at high levels in the colon (12, 16, 20), but they have different spatial distributions. Whereas NHE3 mRNA and protein seem to be present almost exclusively in surface cells (2, 7, 9, 12, 16), NHE2 mRNA and protein do not only occur in surface cells but extend downward into the crypt (2, 7) and possibly occur also at the crypt base (2, 7, 9, 12, 16). In mouse colonocytes, robust apical Na\(^+/\)H\(^+\) exchange has been detected near the base of colonic crypts (9). This activity is stimulated by short-chain fatty acids in the luminal fluid and could play a role in the absorption of these acids (9). Pharmacological studies of isolated crypt cells or of crypts in intact epithelial sheets have suggested that NHE2 best matches the identified transport activity in both proximal and distal colonic crypts of wild-type mice (2, 9). This view is supported by immunostaining showing NHE2-like epitopes near the crypt base (7, 9). However, these data must be interpreted with caution. In isolated colonic crypt cells, Na\(^+/\)H\(^+\) exchange activity was similar between wild-type and NHE2-null mice (2, 13), indicating that the loss

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of NHE2 did not significantly impair total Na⁺/H⁺ exchange. It was suggested that the observed twofold increase in NHE3 mRNA may be part of a compensatory response in NHE2-null mice that explains why eliminating NHE2 did not affect net Na⁺ transport (2). However, in this system, it was not possible to determine whether the observed transport activity was located at the apical membrane or whether NHE3 was upregulated in the crypt cells that normally express NHE2.

The purpose of the present study was to determine whether NHE2 mediates apical Na⁺/H⁺ exchange near the base of mouse colonic crypts. To explore this possibility, we employed a system in which distal colonic epithelium from wild-type mice and NHE knockout mice (as negative controls) are mounted in a chamber that allows independent superfusion of both mucosal and serosal solutions while simultaneously imaging pH$_i$ in colonic epithelial cells by laser scanning confocal microscopy. These experiments, along with immunofluorescence cytochemistry, allowed us to unambiguously evaluate 1) the expression and activity of NHE2 and NHE3 in colonic crypts and at the colonic surface; 2) the effects of NHE2 disruption on both apical Na⁺/H⁺ exchange and the distribution of NHE2- and NHE3-immunoreactive epitopes; and 3) the possibility that either NHE3 or paracellular Na⁺ leakage, possibly in combination with basolateral NHE1 activity, mediates the pH$_i$ response to luminal Na⁺ stimulation near the crypt base. The results provide the first conclusive evidence of apical NHE2 activity in the colon, show that NHE2 is the major apical NHE in colonic crypts, and demonstrate that NHE3 is not the Na⁺-free solution, tetramethylammonium chloride (TMA-Cl) replaced all NaCl (mol:mol), whereas in the ammonium medium, 25 mM NH₄Cl replaced equimolar NaCl or TMA-Cl. The NHE blocker EIPA was from Sigma (Natick, MA). HOE694 and S-1611 (generous gifts from Dr. H. J. Lang, Aventis Pharma Deutschland, Frankfurt/Main, Germany) were used to inhibit NHE2 and NHE3, respectively. 4-Acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS; Research Organics, Cleveland, OH) was used to test for the presence of the Na⁺-HCO$_3^-$ cotransporter. All solutions were prepared directly before use and adjusted to pH 7.4. In some cases, 100 µM lucifer yellow (LY; CH lithium salt, Molecular Probes) was added to superfusates.

Confocal microscopy measurement of pH$_i$. Confocal microscopy was carried out as described previously (10) with modifications. Briefly, for studying colonic crypts, tissue was loaded with BCFEC, and ratiometric measurements of dye fluorescence (alternating 488/458-nm excitation, >505-nm emission) were performed to assess the pH$_i$ while tissue was focused near the crypt base. The plane of focus was selected to be directly adjacent to the base of crypts such that the crypt lumen was just visible and crypt epithelial cells within the image could be visualized along their apical-to-basal pole (9). We used 60% of 458-nm laser power and 3% of 488-nm laser power for BCFEC, and each image was captured in ~3 s. No photobleaching was observed, even after imaging of the same region for 120 min (data not shown). At the end of the time-course experiments measuring pH$_i$, imaging parameters were switched to measure a time course of LY fluorescence in the same region (458-nm excitation, 620- to 680-nm emission), avoiding optical contamination by BCFEC. Membrane-impermeant LY (100 µM) was added sequentially to luminal and then luminal plus serosal perfusates to quantify transepithelial LY leakage. The routine measurement of transepithelial leakage was made when LY was only added to the luminal perfusate. In this condition, the average intensity of LY in the lumen was divided by the intensity of LY in the lamina propria adjacent to the crypt (avoiding areas where LY had bound to connective tissue). This LY ratio provided a measure for the degree of egress of LY from the lumen to serosa (e.g., a LY ratio of 1 means equilibrium). To measure colonic surface epithelial cells loaded with SNARF-5F, fluorescence was excited at 543-nm excitation (with 15% of the laser power) and 550- to 600-/620- to 680-nm emission wavelengths measured simultaneously. Images were analyzed using Metamorph software (Universal Imaging, Downingtown, PA).

Immunocytochemistry. Chemical tissue fixation, sectioning, immunostaining, and confocal imaging of NHE2 and NHE3 epitopes were performed exactly as described previously (10), but the antiserum to NHE2 was used in 1:100 dilution and that to NHE3 in 1:900 dilution. The polyclonal antiserum against NHE2 (rat NHE2 amino acids 676–813) and NHE3 (rat NHE3 amino acids 528–648) had been produced in rabbits (kindly provided by Drs. E. B. Chang and M. Musch, University of Chicago, Chicago, IL). Preimmune sera (by Drs. E. B. Chang and M. Musch) were used in 1:100 dilution. Secondary antiserum was used in 1:400 dilution (Alexa 488-labeled goat anti-rabbit IgG, Molecular Probes).

Statistics. Data between two groups were compared using the two-tailed paired or unpaired Student’s t-test. Differences were considered to be statistically significant at α = 5%. Data are expressed as
means ± SE. Data statistics were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

RESULTS

Apical Na⁺/H⁺ exchange activity near the base of NHE2-null mice distal colonic crypts. Previously, inhibitor profiles suggested that NHE2 is the most likely candidate for an apical NHE in colonocytes near the base of colonic crypts (9). These studies used confocal microscopy to report the pHi from intact epithelial sheets of the normal mouse distal colon. Previous use of NHE2 knockout animals to assess NHE2 functions in colonic crypts had been restricted to a study of isolated crypt colonocytes of uncertain location along the crypt-to-surface axis from the proximal colon (2). We therefore used confocal microscopy to first compare the response to luminal Na⁺ in NHE2-null versus wild-type mice. An NH₄Cl prepulse was used to acidify colonocytes, and a transition from Na⁺-free (TMA containing) medium to 140 mM Na⁺ medium in either the luminal or serosal perfusate was used to activate apical or basolateral Na⁺/H⁺ exchange, respectively, leading to the recovery of resting pHi. A second round of acidification and Na⁺ return was carried out, confirming that pHi recovery was robust and reproducible and that any potential photodamage to tissue was not sufficient to alter cell function in this setting.

As shown, the colonocytes near the crypt base from wild-type (Fig. 1A) and NHE2-null mice (Fig. 1B) manifested a qualitatively and quantitatively similar pHi recovery in response to luminal Na⁺ addition. There were no significant differences in the initial rates of pHi recovery (ΔpHi/min) from the nadir pHi, either between genotypes or between the first and second round of pH recovery (wild-type mice: first round, 0.075 ± 0.011, n = 15 crypts from 2 animals; second round, 0.067 ± 0.007, n = 15 crypts; NHE2-null mice: first round, 0.077 ± 0.006, n = 16 crypts from 2 animals; second round, 0.077 ± 0.015, n = 16 crypts). The nadir pHi after acidification was not different between wild-type mice (6.06 ± 0.04, n = 37 crypts from 5 animals) and NHE2-null mice (6.11 ± 0.03, n = 37 crypts from 5 animals). Similarly, the resting pHi was not different between wild-type mice (6.94 ± 0.03, n = 37 crypts) and NHE2-null mice (7.11 ± 0.05, n = 37 crypts). Therefore, surprisingly, no difference between wild-type and NHE2-null mice was detected at this level.

Apical pHi recovery after the addition of luminal Na⁺ was characterized in more detail between the two genotypes. Because the rate of Na⁺/H⁺ exchange is a strong function of pHi (1), we compared the pHi dependence of the initial pHi recovery rates between genotypes. As shown in Fig. 2A, both genotypes revealed the expected correlation between the extent of acidification and observed rate of pHi recovery, with no readily identifiable difference between genotypes. To compare the initial pHi recovery rate of the two genotypes over a smaller pH range, data were subdivided into two pH ranges (above and below pH 6.1). When average rates of pHi recovery were compared (Fig. 2B), there was no significant difference between genotypes.

In NHE2-null mice, the apical pHi recovery responses of base crypt cells to a number of NHE inhibitors were investigated. The NHE2 inhibitor HOE694 (20 μM) effectively blocked 68% of the apical pHi recovery in response to luminal Na⁺ in wild-type mice and had no effect on pHi recovery in
NHE2-null mice (Fig. 3). Previously, we have shown that the NHE3 inhibitor S-1611 (20 μM) had no effect on apical pH recovery in wild-type mice (9). Here, we demonstrate that S-1611 also did not affect this recovery in NHE2-null mice (Fig. 3), which strongly suggests that NHE3 is not functional at the base of the colonic crypt in either genotype. Finally, the general NHE inhibitor EIPA (20 μM) blocked 74 ± 3% (n = 37 crypts from 5 animals, P < 0.001) of the pH recovery in response to luminal Na+ addition in wild-type mice. However, neither HOE694 nor 20 μM S1611 (NHE3 inhibitor) inhibited the apical Na+ recovery rate in NHE2-null mice. Data are presented as means ± SE; n indicates the numbers of crypts in each group with data from 7 animals. *P < 0.05 vs. control (no inhibitor added) in the same genotype.

46 ± 8% (n = 37 crypts from 3 animals, P < 0.001), was significantly (P < 0.05) less than in wild-type mice, suggesting that both an EIPA-sensitive and -insensitive compensatory mechanism may be involved.
Although experiments were performed in the absence of added CO$_2$-HCO$_3^-$, atmospheric CO$_2$ may be sufficient to activate HCO$_3^-$ transporters. To test the possibility that a HCO$_3^-$ transporter (e.g., Na$^+$-HCO$_3^-$ cotransporter) compensates for the lack of NHE2, the effect of 1 mM SITS was evaluated. SITS did not inhibit the pH$_i$ recovery rate in NHE2-null mice after the addition of luminal Na$^+$ (control: 0.13 ± 0.01 ΔpH$_i$/min and +SITS: 0.12 ± 0.01 ΔpH$_i$/min, n = 40 crypts from 3 animals, P > 0.05).

These results show that in response to luminal Na$^+$, NHE2-null mice utilize a different mechanism to recover the pH$_i$ rate than wild-type mice and that this mechanism is sensitive to EIPA but unlikely to be NHE3- or SITS-sensitive HCO$_3^-$ transporter.

**Apical Na$^+/H^+$ exchange activity in surface epithelial cells.** Colonic surface epithelial cells, in contrast to crypt cells, have been reported to contain both NHE2 and NHE3 mRNA and protein (2, 6, 7, 9, 12, 16). Therefore, we used measurements of pH$_i$ as a means to compare epithelial function in surface cells versus crypt cells of wild-type mice. Figure 5A shows that the colonic surface could be loaded with the pH sensor SNARF-5F. As shown in Fig. 5B, both 20 μM HOE694 and 20 μM S1611 caused a significant inhibition of the Na$^+$-dependent recovery from an acid load in surface cells (33% or 57% inhibition of transport, respectively). In addition to supporting the presence of both apical NHE2 and NHE3 function in colonic surface cells, the results confirm the efficacy of S-1611 as an inhibitor in our experimental preparation and demonstrate

![SNARF-5F](image)

**Fig. 5.** Measurements of pH$_i$ in colonic surface epithelial cells and cellular responses to specific NHE inhibitors in wild-type mice. A: confocal images of the colonic surface during superfusion and after being loaded with 5 μM SNARF-5F [left, 550- to 600-nm emission (Em), middle, 620- to 680-nm emission; right, transmitted light image]. B: experiments were performed as in Fig. 1, and results were analyzed as in Fig 3. With the use of tissues from wild-type mice, S-1611 or HOE694 (both 20 μM) was added to the luminal compartment after the second NH$_4^+$ prepulse acidification. Initial rates of pH$_i$ recovery in the presence of inhibitor were normalized to the initial rate of transport (ΔpH$_i$/min) observed in the absence of inhibitor from the same crypt (control = 100%). Results are presented as means ± SE for n = 5 tissues from 4 animals. *P < 0.05 vs. control; **P < 0.01 vs. control. Scale bars = 20 μm.
striking functional differences between cells at the surface versus cells at the crypt base.

Possible role of NHE1 and transepithelial Na⁺ leakage. A notable weakness of using pHᵢ measurements is that inadvertent activation of basolateral Na⁺/H⁺ exchange may confound observations. This has been partially addressed in our prior work (9) using normal mice. However, if NHE2-null mice had leakier crypts, the sustained pHᵢ recovery could be explained by Na⁺ leaking to the serosal compartment and activating basolateral Na⁺/H⁺ exchange. This is especially important given that both HOE694 and EIPA are effective inhibitors of NHE1. We therefore used the transepithelial leakage of LY from the luminal to serosal compartment as a measure of transepithelial Na⁺ leakage. This leakage was measured during the steady-state addition of LY exclusively to the luminal perfusate and quantified as the ratio of luminal to serosal intensity of LY in this condition. We have used such an LY ratio measure previously as a gross assay of tissue leakiness (9). To validate the suitability of the parameter more rigorously, we used the observation that individual crypts varied significantly in this ratio (range 3–35). One predicted reason for a low LY ratio is that mixing of luminal fluid with leaked serosal fluid will decrease the luminal fluorescence. If this prediction is correct, the addition of serosal LY should increase the luminal LY fluorescence most strongly in crypts with a low LY ratio. To test this prediction, we divided observations into “leaky crypts” (defined as LY ratio < 10; mean LY ratio: 5.9 ± 0.3, n = 28 crypts) and “tight crypts” (defined LY ratio > 10; mean LY ratio: 20 ± 1, n = 51 crypts) and then measured the change in luminal intensity when LY was added to only luminal perfusate versus when it was present in both luminal and serosal perfusates. We observed that leaky crypts increased their luminal intensity (149 ± 8%) more than tight crypts (112 ± 3%, P < 0.0001). The results suggest that the LY ratio is a valid parameter for individual crypt solute leakiness and can resolve differences of functional significance.

Fig. 6. Paired measures of pHᵢ and paracellular leakiness in individual colonic crypts. A: pHᵢ determined by ratio imaging using 3 μM BCECF [left, 488-nm excitation (Ex); middle, 458-nm Ex]. Right, intracellular BCECF ratio (488-to-458 nm) in pseudocolor, with the corresponding pHᵢ scale indicated qualitatively by the color bar. B: at the end of each experiment, 100 μM of the fluorescent, membrane-impermeant lucifer yellow (LY; 458-nm Ex and 620- to 680-nm Em) was added to luminal and then to luminal + serosal perfusates to quantify transepithelial LY leakage. As shown, LY could be imaged without optical contamination by BCECF. In the presence of only luminal LY, the LY ratio was assessed as the intensity of LY in the lumen divided by the intensity of LY in the lamina propria adjacent to the crypt (S), providing a measure of LY egress from the lumen to serosa. *Cross-sectioned lumen near the crypt base. Scale bars = 20 μm.
We next used the LY ratio to test whether enhanced trans-epithelial leakage of luminally added Na\(^+\)/H\(^+\) would stimulate basolateral Na\(^+\)/H\(^+\)/H\(^+\) exchange activity more in NHE2-null mice than in wild-type mice. This required us to run a pH\(_i\) experiment and then afterward evaluate individual crypt LY ratios for the same crypts, so that rates of transport could be directly correlated. Figure 6 demonstrates that LY fluorescence could be measured completely separately from BCECF fluorescence. Figure 6A shows colonic crypts loaded with BCECF, whereas Fig. 6B reveals the same crypts after LY had been added to perfusates.

Using these combined measures, we first found that the LY ratio did not differ significantly between wild-type mice (15 ± 2, n = 27 crypts from 5 animals) and NHE2-null mice (17 ± 1, n = 33 crypts from 5 animals), suggesting that there is no general change in paracellular leakiness between the genotypes. Furthermore, when individual crypt LY ratios versus the rate of pH\(_i\) recovery were directly plotted, there appeared to be no obvious negative correlation between the LY ratio and pH\(_i\) recovery rate in response to luminal Na\(^+\) (wild-type mice: \(R^2 = 0.07\), NHE2-null mice: \(R^2 = 0.11\); Fig. 7). These results indicate that 1) crypts in NHE2-null mice are not more leaky than those in wild-type mice, and 2) transepithelial leakage of Na\(^+\) does not affect the apical pH\(_i\) recovery rate of NHE2-null mice any differently (if at all) from wild-type mice.

The LY ratio can report paracellular solute leakiness but may be a weak indicator of paracellular Na\(^+\) leak or transcellular Na\(^+\) flux in Na\(^+\)-absorbing tissue. Therefore, to extend our observations, we used NHE1-null mice, which lack the NHE1 isoform [presumed to be the basolateral NHE of colonic crypts (4)]. As shown in Fig. 8A, crypt colonocytes still underwent pH\(_i\) recovery in response to luminal Na\(^+\) but exhibited no response to serosal Na\(^+\). These results confirm the assumption that NHE1 is the only basolateral NHE in the mouse colonic crypt and show that our results after luminal Na\(^+\) load cannot be explained by transepithelial Na\(^+\) flux that subsequently would have activated basolateral NHE1. The pH\(_i\) recovery rate in response to luminal Na\(^+\) for NHE1 mutants (0.040 ± 0.003...

Fig. 7. Correlation of paracellular leakiness versus the pH\(_i\) response to luminal Na\(^+\). No correlation was apparent between the LY ratio (lumen/serosa) and apical pH\(_i\) recovery rate (\(\Delta\)pH/min) for either wild-type mice (\(R^2 = 0.07\), n = 27 crypts from 5 animals) or NHE2-null mice (\(R^2 = 0.11\), n = 33 crypts from 5 animals).
ΔpH/min, n = 33 crypts from 6 animals) did not differ significantly from that of wild-type mice. As shown in Fig. 8B, 20 μM luminal HOE694 inhibited the apical pH recovery rate by 75% (0.009 ± 0.002 ΔpH/min, n = 21 crypts from 4 animals), which was similar to the level of inhibition observed in wild-type mice.

Localization of NHE2 and NHE3 proteins in the mouse colonic crypt and surface. The lack of effect of a NHE3-specific inhibitor on crypt cell pH recovery was surprising given the prior suggestion that upregulation of NHE3 compensated for the absence of NHE2 (2). Therefore, we sought to confirm that the epitope previously identified at the crypt base with an anti-NHE2 antiserum was truly NHE2 and compared the localization of NHE3 between wild-type and NHE2-null mice. Immunofluorescence staining with the anti-NHE2 antiserum showed strong immunoreactivity in wild-type mice (Fig. 9A) but not in NHE2-null mice (Fig. 9B), confirming the reliability of the antiserum and greatly strengthening the conclusion that NHE2 is present near the crypt base in wild-type animals. Preimmune serum (Fig. 9, C and D) also exhibited no immunoreactivity. In the distal colon of wild-type mice, NHE2 was observed in the apical brush border of colonocytes and throughout the crypts, from the crypt surface to the crypt base, but with highest intensity at the surface and at the base, as seen previously (9).

Using immunofluorescence staining, we also assessed the presence and distribution of NHE3. NHE3 immunoreactivity was present in the brush border of surface colonocytes in both wild-type mice (Fig. 10A) and NHE2-null mice (Fig. 10C). The specificity of immunostaining was confirmed by the lack of staining in the distal colon of NHE3-null mice (Fig. 10E), which served as a negative control, and with the use of NHE3 preimmune serum (Fig. 10, B, D, and F). To appraise potential differences in NHE3 distribution in the crypts of NHE2-null mice, we used a semiquantitative grading scale and evaluated the intensity of the immunofluorescence in different regions of the crypt. With the use of confocal images of longitudinally sectioned crypts, the total crypt was subdivided into four equal regions, from surface to base: region A, surface area; region B, upper crypt; region C, middle crypt; and region D, crypt base. In each region, the intensity of the fluorescence was scored as strong, weak, or absent; the results are shown in Table 1. These results demonstrate that in NHE2-null mice, NHE3 staining extended deeper into the crypt than in wild-type mice and also confirmed that the crypt base was always immunonegative. The immunostaining results suggest a possible increase in NHE3 protein that may correspond to the previously reported increase in NHE3 mRNA in NHE2-null mice (2).

DISCUSSION

NHE isoforms are involved in intestinal Na+/H+ transport, but the functions of individual NHEs in different parts of the intestine of various mammalian species are under debate (e.g., Ref. 28). Here, we report on the distribution and function of NHE2 and NHE3 proteins in the mouse distal colon. It was previously suggested, but not proven, that NHE2 is important to apical Na+/H+ exchange in the murine colonic crypt. In the
In the present study, we used wild-type and NHE-null mice in a preparation that allowed direct visualization of apical NHE function in crypts. We provide evidence that NHE2 is the main apical NHE near the base of the crypts, because 1) specific NHE2 immunoreactivity is present, 2) HOE694-sensitive exchange activity occurs in wild-type mice but is absent from NHE2-null mice, and 3) this activity accounts for greater than two-thirds of the total response to luminal Na\(^{+}/\)H\(^{+}\). On the basis of combined use of pharmacological and genetic approaches applied to intact epithelial structures, this is the first direct demonstration of apical Na\(^{+}/\)H\(^{+}\) uptake activity in the colon that can be unequivocally attributed to NHE2. In the kidney, such activity has been recently shown by Bailey et al. (3). Using a modification of our protocol to study pH\(_i\) in crypt epithelial cells, we were able to confirm that cells at the colon surface revealed a different inhibitor profile, suggesting the functional presence of both NHE2 and NHE3. This prominent role of NHE2 at the surface as well as in the crypt may explain why this transporter is expressed at a high level in the colon.

Transepithelial Na\(^{+}\) leakage does not contribute to pH\(_i\) recovery in wild-type or NHE2-null mice. Previously, we identified dye leakage between the luminal and serosal compartment in our preparation of mouse colonic sheets (9). This raised the possibility that transepithelial leakage of Na\(^{+}\) from the mucosal to serosal solution activates basolateral NHE1 activity to become part of the colonocyte pH\(_i\) recovery response to luminal Na\(^{+}\) addition. Obviously, such a situation would confound reliable assessment of the activity of apical Na\(^{+}/\)H\(^{+}\) exchange. This possibility might be tested by pharmacologically inhibiting NHE1 activity, but in our hands, none of the available blockers fully inhibited NHE1 in colonic...
epithelium without partially affecting other NHE isoforms as well (Y. Guan, unpublished observations). Here, we tested the influence of transepithelial Na\(^+\) leakage using NHE1-null mice and LY as a marker for paracellular leakage. We conclude that basolateral NHE1 activity is not activated by transepithelial leakage of luminally applied Na\(^+\), for the following three reasons. First, by checking the LY ratio between the luminal and serosal compartment before the experiment, we were able to discard preparations with intercompartmental leakage due to improperly mounted colonic sheets in the superfusion chamber. Second, assuming that a low LY ratio indicates strong paracellular leakage of LY, and presumably also of Na\(^+\), one would expect to find that low LY ratios would be negatively correlated to high, Na\(^+\)-stimulated NHE1 activity and, hence, to a high pH\(_i\) recovery rate. However, we did not observe a correlation between the LY ratio and pH\(_i\) recovery rate. This indicates that paracellular leakage, although measurable, does not flux enough Na\(^+\) into the basolateral spaces to influence the response to luminal Na\(^+\). Finally, the use of NHE1-null mice enabled us to completely eliminate basolateral NHE activity. Because the mutants showed a normal HOE694-sensitive pH\(_i\) recovery rate upon luminal Na\(^+\) addition and no substantial Na\(^+\)/H\(^+\) exchange during basolateral Na\(^+\) perfusion, an apical Na\(^+\)-dependent acid extrusion mechanism must be present.

Importantly, we found that in the absence of NHE2, another apical Na\(^+\)-dependent acid extrusion mechanism was upregulated. Using NHE2-null mice, Bachmann et al. (2) concluded that mutating the NHE2 gene did not diminish total Na\(^+\)/H\(^+\) exchange activity in cells isolated from the mouse proximal colon. The present study extends this conclusion to the distal colon, because it shows a robust and reproducible pH\(_i\) recovery in colonocytes near the base of the distal colonic crypt, with a rate that did not differ between wild-type and NHE2-null mice. On the basis of RT-PCR, Bachmann et al. (2) found that isolated cells from colonic crypts have an increased expression of NHE3 mRNA in NHE2-null mice and suggested this as the compensatory mechanism sustaining transport activity (2). We extend this observation to the protein level, revealing by immunocytochemistry that NHE3 protein in NHE2-null mice extends deeper into crypts. However, no NHE3 protein was detected at the base of the crypt, and the NHE3 blocker S-1611 was ineffective as an inhibitor of pH\(_i\) recovery in response to luminal Na\(^+\) in the crypts of NHE2-null mice (although it was an effective inhibitor of transport in surface cells). Based on these observations, we conclude that NHE3 is unlikely to be the apical Na\(^+\)-dependent acid extrusion mechanism that acts in the crypts of NHE2-null mice. Because EIPA, which inhibits most known NHE isoforms (20), blocks >50% of the apical pH\(_i\) recovery in NHE2-null mice, one candidate for this apical extrusion mechanism is another NHE isoform that is neither NHE3 nor NHE1. Because EIPA may also inadvertently affect non-NHE transporters, the results might also be explained by the action of an apical Na\(^+\)-HCO\(_3\) cotransporter (23). However, the lack of effect of the HCO\(_3\)\(_{-}\) transport inhibitor SITS (5, 22) on pH\(_i\) recovery in the crypts of NHE2-null mice does not support this possibility. Alternatively, a combination of apical transporters that are indirectly linked (e.g., via pH or membrane potential changes) to evoke Na\(^+\)-dependent net acid extrusion may play a role, because the significantly smaller effect of EIPA in NHE2-null mice suggests that multiple compensatory mechanisms, both EIPA sensitive and insensitive, may be involved. Irregardless, EIPA will be a useful tool for further exploration of the nature of at least one component of this upregulated transport.

NHE2-null mice do not show diarrhea, leading investigators to previously conclude that NHE2 does not play an important role in the absorption of Na\(^+\) and water in the colon of the normal mouse. However, previous data as well as our present observations indicate that lack of NHE2 leads to a compensatory response to replace the missing functionality of NHE2 in crypts (2). Because we have identified this compensatory response as apical transport activity, the possibility remains that NHE2 mediates at least a fraction of Na\(^+\) absorption in normal animals and that this role is replaced by an alternative mechanism in NHE2-null mice. It must be noted that, although crypts have been shown to mediate Na\(^+\)-dependent fluid absorption (15, 26), at present there is no direct evidence that colonic crypts absorb Na\(^+\). However, the presence of a compensatory response to functionally replace an absent NHE2 provides compelling evidence that NHE2 serves a vital function in the crypts. Identifying the constellation of Na\(^+\)-uptake mechanisms in the mouse colonic crypt, their relevance to net Na\(^+\) absorption, and the identity of the compensatory mechanism(s) caused by the absence of NHE2 are important goals of future research.

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