Electroneutral sodium absorption and electrogenic anion secretion across murine small intestine are regulated in parallel

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The coordination of transepithelial anion secretion and NaCl absorption across small intestinal epithelia contributes to the balance between systemic fluid homeostasis and hydration of the luminal contents. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
severe intestinal impactions (6, 12, 44). Similar to CFTR-null (CFTR(−)) mice, gene-targeted disruption of the Na⁺-K⁺-2Cl⁻ co-transporter (NKCC1) results in reduced anion secretory capacity and moderate intestinal pathology with an increased incidence of fatal intestinal impactions (16). Loss of NKCC1 activity likely results in fewer fatal impactions than loss of CFTR activity, because the NKCC1(−) intestine retains electrogenic HCO₃⁻ secretion and a residual amount of electroneutral Cl⁻ secretion mediated by an alternate Cl⁻ uptake pathway across the basolateral membrane, possibly AE2 (50).

Adaptation to disruptions in intestinal ion transport in these mouse models may include parallel changes in the opposing pathway to moderate the severity of intestinal disease and lessen systemic fluid imbalance. In the present study, we hypothesized that disruption in either the absorptive or secretory pathway would result in diminished activity of the opposite process, thereby reducing the risk of systemic fluid imbalance. In the present study, we hypothesized that disruption in either the absorptive or secretory pathway would result in diminished activity of the opposite process, thereby reducing the risk of systemic fluid imbalance.

**MATERIALS AND METHODS**

**Animals**

The experiments in this study used mice with gene-targeted disruptions of either NHE3 (Slc9a3), NKCC1 (Slc12a2), or CFTR (cfr牵手m1转基因) strain with defined backgrounds, as described previously (16, 39, 44). In some studies, mice homozygous for the ΔF508 CFTR mutation (cfr牵手m1转基因) strain were used (49). All comparisons were made with gender- and age-matched (+/+ or −/−) siblings (WT). As previously reported (10), mice at 3–6 wk of age were genotyped and identified as WT, NHE3(−), CFTR(−), homozygous ΔF508 CFTR (CFTR[ΔF/ΔF]), or NKCC1-null [NKCC1(−)]. All mice were maintained on standard laboratory chow (Formulab 5008, Nestle Purina, St. Louis, MO or Rat/Mouse Diet 7012, Harlan Teklad, Madison, WI). To prevent intestinal impaction in the CF mice, an oral osmotic laxative (polyethylene glycol 3350 MW) was provided in the drinking water of the CF mice and their WT littermates (10); all other mice were maintained on standard laboratory chow (Formulab 5008, Nestle Purina, St. Louis, MO or Rat/Mouse Diet 7012, Harlan Teklad, Madison, WI). To prevent intestinal impaction in the CF mice, an oral osmotic laxative (polyethylene glycol 3350 MW) was provided in the drinking water of the CF mice and their WT littermates (10); all other mice were maintained on standard laboratory chow (Formulab 5008, Nestle Purina, St. Louis, MO or Rat/Mouse Diet 7012, Harlan Teklad, Madison, WI).

**Bioelectric Measurements**

Animals were killed by asphyxiation in a 100% CO₂ atmosphere followed by a surgical thoracotomy to induce pneumothorax. Proximal small intestine (~5 cm proximal jejunum) was removed via an abdominal incision and immediately placed in an ice-cold, oxygenated Ringer solution. Intestinal segments were opened along the mesenteric border using sharp dissection and sectioned before mounting full thickness in standard Ussing chambers (0.238-cm² surface area), as previously described (12). In some experiments as indicated, the outer intestinal musculature was blunted dissected and removed before study. Mucosal and serosal surfaces of the intestinal segments were bathed with 4 ml of warmed (37°C), oxygenated Krebs bicarbonate Ringer solution with the following composition (in mM): 140 Na⁺, 119.8 Cl⁻, 25 HCO₃⁻, 5.2 K⁺, 2.4 HPO₄⁻, 0.4 H₂PO₄, 1.2 Mg, and 1.2 Ca, pH 7.4. Glucose (10 mM) was added to the serosal bath, and mannitol (10 mM) was added to the mucosal bath to avoid an inward current due to Na⁺-coupled glucose co-transport (11). All intestinal segments were treated with indomethacin (1 µM) and TTX (0.1 µM) to minimize the effect of endogenous prostaglandins and neural tone, respectively, as previously described (9, 12, 42). Transepithelial Iₜₑ (µA/cm²) was measured using an automatic voltage-current clamp (VCC-600, Physiologic Instruments, San Diego, CA) as previously described (11). Transepithelial conductance (Gₑ; ms/cm²) was determined using Ohm’s law by applying a 5-nA pulse and measuring the resulting current deflection.

**Radioisotopic Fluxes Measurements**

Triplicate 250-µl samples taken at the beginning and end of each 30-min flux period (following a 30-min equilibration period) were used to calculate unidirectional mucosal-to-serosal (Jₑ) fluxes of 22Na in tissue pairs treated with vehicle (DMSO) or EIPA as previously described (9, 21, 40). EIPA-sensitive Jₑ⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺(506,870),(973,997)
from CFTR(−), CFTR(ΔF/ΔF), NHE3(−), NKCC1(−) mice, or their WT littermate controls.

Antibodies. The rabbit polyclonal antibody R3195 was raised against a thyroglobulin-conjugated 13-amino acid COOH-terminal peptide sequence of rodent CFTR, affinity-purified on a peptide-epoxide-activated Sepharose column, eluted with 4.9 M MgCl2, dia-lyzed, and concentrated. CFTR-labeling specificity has been demonstrated in Western blot analysis and immunocytochemical assays by the loss of immunostaining in tissue specimens from CFTR(−) mice (17). The affinity-purified anti-human polyclonal anti-NHE3 antibody (Chemicon) was raised against a 22-amino acid peptide within the cytoplasmic, COOH-terminal domain of the human NHE3, coupled to keyhole limpet hemocyanin. The band intensities of the immunostained NHE3 (~80 kDa) and CFTR C-band (~180 kDa) showed considerable deviation from linearity below a threshold level (band intensity decreased to a greater extent than would be predicted from dilution of the protein concentration). Therefore, band intensity signals for NHE3 and CFTR C-band were calibrated by running serial dilutions of a NHE3- and CFTR-enriched sample (i.e., purified mouse intestinal brush-border membranes) on the same gel and then correcting the measured intensity of each band for nonlinearity. The average values stated in the figures and figure legends are based on the relative amounts of NHE3 or CFTR C-band protein calculated from the scanned band intensities and the calibration plot.

Epithelial Morphometry

Isolated jejunal segments were fixed using 1.25% glutaraldehyde, 1% paraformaldehyde, 130 μM CaCl2, and 130 mM sodium cacodylate. Each fixed sample was cut in half, dehydrated by a graded series of ethanol, and transferred to propylene oxide before being embedded in Epon-Araldite (Electron Microscopy Sciences, Ft. Washington, PA). Fixed tissues were cut into two sets of ten 1-μm serial sections with 250 μm between the two sets. All sections were stained with toluidine blue and examined by light microscopy.

Serial sections were examined at ×400 magnification for villi with the largest diameter and full-length crypts using an upright microscope (Olympus BX50WI, Tokyo, Japan). Candidate villi and crypts were photographed using a SensiCam digital camera ( Cooke, Auburn Heights, MI), and morphological measurements of villus height, crypt depth, and epithelial cell number were obtained using ImagePro Plus (Media Cybernetics, Carlsbad, CA). Epithelial cell number was determined by counting the number of nuclei in a section of villus or crypt and normalizing to the length of the epithelial cell basement membrane. Villus epithelial cell area was measured as previously described (20).

Statistics

An unpaired Student’s t-test assuming equal variances was used to compare data between two treatment groups. A probability value of P < 0.05 was considered statistically significant. All values are reported as means ± SE.

Materials

Radioactive sodium (22Na) was obtained from New England Nuclear (Boston, MA). EIPA was obtained from Sigma-RBI (Natick, MA). Unless stated otherwise, all other reagents were obtained from either Sigma (St. Louis, MO) or Fisher Scientific (Springfield, NJ).

RESULTS

Reduced Anion Secretion in NHE3(−) Intestine

NHE3(−) mice, in which the dominant pathway for electroneutral Na+ absorption is disrupted, exhibit chronic diarrhea and evidence of perturbed systemic fluid balance (39). To determine whether the reduced absorption across NHE3(−) intestine results in a compensatory decrease in transepithelial secretion, peak Isc and bumetanide-sensitive Isc were measured during cAMP stimulation (treatment with 10 μM forskolin/100 μM IBMX) of small intestine from NHE3(−) mice and their WT littermates. As shown in Fig. 1A, NHE3(−) jejuna exhibited significant reductions in both peak and bumetanide-sensitive Isc responses, suggesting reduced CFTR-mediated anion secretion in the absence of NHE3. However, NHE3(−) jejuna also exhibited significantly reduced Gs compared with WT (Fig. 1B). In a leaky epithelium such as the small intestine, Gs is largely a measure of the conductance through the paracellular “shunt” pathway (18), which is composed of series conductance through both the tight junction and the dynamic fluid compartment of the lateral intercellular spaces (LIS) (5, 28). We have recently shown (19) that the patency of the LIS (as indexed by Gs) is a positive predictor of the magnitude and duration of the anion secretory response to cAMP stimulation in the murine small intestine. Thus a reduced Gs in the NHE3(−) intestine might limit the magnitude of the Isc response to forskolin/IBMX. First, to determine whether the relationship between Gs and the cAMP-stimulated Isc in the NHE3(−) intestine was similar to the positive relationship recently demonstrated between these parameters in WT intestine (19), basal Gs was plotted against the peak ΔIsc following increased cAMP. As shown in Fig. 1C, NHE3(−) intestine exhibited a significant positive correlation between baseline Gs and the Isc response to cAMP (n = 56). Next, to eliminate differences in Gs between NHE3(−) and WT intestine, the outer musculature of the intestine was removed (i.e., muscle stripped), which sustains the patency of the LIS in murine intestinal epithelium (19). As shown in Fig. 2A, Gs was similar between NHE3(−) and WT intestine under basal and stimulated conditions. However, as shown in Fig. 2B, the cAMP-stimulated ΔIsc and bumetanide-sensitive Isc were still reduced in the NHE3(−) intestine compared with WT intestine.

A second possibility for reduced anion secretion across the NHE3(−) small intestine is a reduced number of cells contributing to transepithelial secretion (i.e., decreased cells/crypt or decreased crypt depth). To evaluate this possibility, morphology of crypt depth and the number of crypt cells per micrometer of basement membrane were measured in WT and NHE3(−) small intestine. As shown by comparison with WT intestine (Fig. 3A), crypt depth was significantly increased in NHE3(−) small intestine and no changes in the number of crypt cells per micrometer of basement membrane were apparent. Increased crypt depth is the opposite of the expected result if anion secretion is decreased as a result of decreased crypt cell number.

A third possibility to account for the decreased secretory capacity of NHE3(−) intestine was downregulation of CFTR protein expression. Previously, it was shown that mRNA expression of CFTR is reduced by ~35% in NHE3(−) small intestine (54). Therefore, CFTR protein expression was evaluated in NHE3(−) and WT small intestine by Western blot analysis. Despite the presence of increased crypt depth in the NHE3(−) intestine, CFTR expression was reduced by 56% in NHE3(−) small intestine compared with WT (see Fig. 3B).
Reduced Na⁺/H⁺ absorption in CFTR(−) and NKCC1(−) intestine

To investigate whether chronic deficiencies in anion secretion result in a parallel decrease in electroneutral Na⁺ absorption, EIPA-sensitive ²²Na absorption (an index of NHE activity) was measured in two murine models with disruptions in the anion secretory pathway: 1) CFTR(−) mice, which lack cAMP-stimulated anion secretion across the intestinal epithelium (44); and 2) NKCC1(−) mice, which lack the dominant mechanism for Cl⁻ uptake across the basolateral membrane but maintain ~50% of WT secretory capacity due to electronegenic HCO₃⁻ secretion and Cl⁻ secretion via an alternative pathway (16). Because previous studies with NHE3(−) intestine had revealed that an EIPA-sensitive, electroneutral Na⁺ transport process (perhaps NHE2) partially compensates for loss of the EIPA-resistant NHE3 (21), it is possible that changes in NHE3 activity in the secretory-deficient intestine may be compensated by increased activity of the EIPA-sensitive Na⁺ transport process. Therefore, we evaluated the sensitivity of electroneutral Na⁺ absorption to both 1 and 100 μM EIPA in the CFTR(−) and NKCC1(−) intestines.

As shown in Fig. 4A, there were no obvious differences in the 1 μM EIPA-sensitive Na⁺ absorption between WT and...
CFTR(−) jejuna, indicating that the aforementioned compensation in Na+ absorption was not present. However, consistent with the hypothesis that chronic disruption of anion secretion results in a compensatory decrease in absorption, CFTR(−) jejuna exhibited a significant reduction in the 100 μM EIPA-sensitive Na+ absorption [indicative of NHE3 activity (21)] compared with WT jejuna. In the NKCC1(−) intestine (see Fig. 4B), it was also apparent that the principal mode of Na+ absorption was not altered because 22Na absorption was not sensitive to 1 μM EIPA. However, similar to findings in the CFTR(−) intestine, the rate of electroneutral Na+ absorption sensitive to 100 μM EIPA was significantly reduced in the NKCC1(−) compared with WT intestine.

To investigate differences in electroneutral Na+ absorption in the CFTR(−) and NKCC1(−) jejuna, we examined villus morphology and the levels of NHE3 protein expression. In morphometric studies, villus height and the number of cells per micrometer of villus basement membrane were measured but no differences from WT intestine were found for either CFTR(−) intestine (Fig. 5A) or NKCC1(−) intestine (Fig. 5B). However, immunoblot studies of NHE3 indicated that protein expression was reduced by 34% in the CFTR(−) small intestine (Fig. 6A). To ensure that changes in NHE3 expression were not specific to the CFTR(−) condition, expression of NHE3 was also evaluated in mice homozygous for the ΔF508 mutation of mCFTR, i.e., CFTR(ΔF/ΔF). In these mice, mutant CFTR protein is translated but the mutant protein is retained in the endoplasmic reticulum and eventually undergoes degradation (43). As shown in Fig. 6B, CFTR(ΔF/ΔF) jejuna exhibited a decrease (26%) in NHE3 protein expression. In contrast to findings in the CFTR(−) and CFTR(ΔF/ΔF) jejuna, however, intestinal epithelia isolated from NKCC1(−) mice exhibited no change in NHE3 protein expression (Fig. 6C) or CFTR protein expression (Fig. 6D). The absence of a detectable change in NHE3 protein expression in the NKCC1(−) intestine indicates that an additional mechanism(s) exists for posttranslational regulation of Na+ absorption in the secretory-deficient intestine.

**DISCUSSION**

Mice with gene-targeted disruptions of either the absorptive or secretory pathway provide powerful tools to explore the regulatory interactions between the processes of absorption and secretion across mammalian intestinal epithelium. Using these animal models, we tested the hypothesis that chronic reductions in absorption or secretion result in downregulation of transport activity in the opposing pathway of transepithelial NaCl movement. Findings that support this hypothesis include 1) gene-targeted disruption of NHE3 resulted in decreased CFTR-mediated anion secretion and reduced CFTR protein expression; 2) gene-targeted disruption of CFTR resulted in
Mean representative mean Na⁺ (19). When LIS volume was normalized by removal of the outer intestinal musculature, anion secretion, as indexed by the $I_{sc}$, was still reduced in the NHE3(−) intestine (Fig. 2). Instead of these possibilities, it is known that CFTR mRNA expression is reduced by 35% in the NHE3(−) murine intestine (54) and, in the present study, it was shown that CFTR protein expression is downregulated by ~50%, indicating that posttranscriptional compensation does not occur. Thus diminution in anion secretory capacity of the intestine in the absence of NHE3 activity may result from reduced expression of CFTR, the rate-limiting step in the mechanism of cAMP$_3$-stimulated anion secretion (14).

The results of the present study show that CFTR mutant and NKCC1(−) mice have significant reductions in NHE3-mediated Na⁺ absorption as measured by the absorptive ²²Na flux sensitive to 100 μM EIPA. The Na⁺ flux measurements in the CFTR(−) intestine differ with an earlier report from this laboratory (12) that had shown that the rate of Na⁺ absorption was similar between CFTR(−) and WT mice. However, this difference is methodological, because our recent studies indicate that the equilibration time for isotopic Na⁺ flux in the serosal-to-mucosal direction across the CFTR(−) intestine requires a longer time period than was used in the earlier report. Furthermore, the reduced rate of 100 μM EIPA-sensitive $J_{msNa}^+$ demonstrated in the CF intestine is supported by a more recent investigation showing that net Na⁺ absorption, as measured by bidirectional ²²Na flux analysis, is reduced in the CFTR(−) intestine (20).

Reduced electroneutral Na⁺ absorption across the intestine of CFTR(−) mice may result from decreased expression of NHE3 protein. As measured by Western blot analysis, NHE3 protein expression in the intestinal epithelium was reduced by 34% in the absence of CFTR expression. NHE3 protein expression was also reduced in the intestinal epithelium from ΔF508 CFTR homozygous mice, in which the mutant protein is translated and partially processed. Thus the characteristic common to both CF models is the loss of CFTR activity in the apical membrane of the villi, which may provide a signal for downregulation of NHE3 expression. Decreased expression of NHE3 protein by both Western blot analysis and immunohistochemical techniques has also been reported in the pancreatic ducts of CF mice (2). Investigation of the recombinant proteins in that study suggests that NHE3 and CFTR form protein complexes involving PSD-95/disc-large/zonula occludens-1 (PDZ) scaffolding proteins; thus the parallel relationship between NHE3 and CFTR expression may involve the relative amounts of each protein bound in these complexes at the plasma membrane. Recent studies (2, 3) support a direct association between CFTR and NHE3, albeit in a reciprocal relationship between the two proteins with regard to cAMP$_3$-regulation of transport activity. This relationship may also involve shared binding of CFTR and NHE3 to PDZ scaffolding proteins, i.e., the Na⁺/H⁺ exchanger-regulatory factor (NHERF) isoforms, which contribute to PKA-dependent regulation of transport activity (51, 52). In addition to parallel changes in the expression of CFTR and NHE3 in the knockout intestines, it is feasible that additional mechanisms that chronically suppress transport activity are operative. One possibility is an alteration in the steady-state distribution of CFTR or NHE3 between the pools in the apical membrane and recycling endosomes (29). Such changes would not be detected in the

decreased NHE3-mediated Na⁺ absorption and decreased NHE3 protein expression; and 3) gene-targeted disruption of NKCC1 resulted in decreased NHE3-mediated Na⁺ absorption.

Previous studies (21, 33, 34) have shown that NHE3 is the major pathway of electroneutral Na⁺ absorption across the small intestine. Mice homozygous for disruption of NHE3 exhibit chronic diarrhea secondary to malabsorption of salt and water (21, 39). In this and a previous study (21), we showed that loss of NHE3 activity in the murine intestine is associated with a decreased rate of transepithelial anion secretion. This change cannot be explained by a stable reduction in the number of crypt epithelial cells available for CFTR-mediated anion secretion, because the crypt length was significantly increased in the NHE3(−) intestine (Fig. 3A). Reduced anion secretion in the NHE3(−) intestine also could not be directly ascribed to stable reduction in the volume of the LIS, which has been shown to be a major determinant of the magnitude and duration of cAMP$_3$-stimulated anion secretion across murine intestine (19). When LIS volume was normalized by removal of the

**Fig. 4.** EIPA sensitivity of Na⁺ absorption across WT, CFTR(−), and Na⁺-K⁺-2Cl⁻ cotransporter-null [NKCC1(−)] mouse small intestine. A: bars represent mean Na⁺ mucosal-to-serosal flux ($J_{msNa}^+$) sensitive to treatment with either 1 or 100 μM EIPA for WT or CFTR(−) intestine (n = 6 pairs). Mean $G_i$ during 100 μM EIPA treatment for WT = 27.8 ± 3.1 and for CFTR(−) = 22.5 ± 1.9 ms/cm² [not significant (NS)]. B: bars represent mean $J_{msNa}^+$ sensitive to treatment with either 1 or 100 μM EIPA for WT or NKCC1(−) intestine (n = 6 pairs). Mean $G_i$ during 100 μM EIPA treatment for WT = 33.2 ± 1.6 and for NKCC1(−) = 35.1 ± 2.0 ms/cm² (NS). *Significantly different from respective WT (P < 0.05).
present study, because the membrane preparations used for immunoblotting likely included endosomal membranes.

The central hypothesis that mechanisms of electrolyte secretion and absorption change in parallel to maintain fluid homeostasis may extend beyond the interactions between NHE3 and CFTR. One consideration is the activity of the Cl⁻/HCO₃⁻ exchanger(s) that are coupled to NHE3 at the apical membrane of villous epithelium, i.e., the exchanger(s) responsible for Cl⁻ absorption. Members of the sulfate permease family of anion exchangers, including downregulated in adenoma (DRA) and putative anion exchange 1 (PAT-1 or CFEX) and the anion exchanger isoform 4 (AE4), are likely candidates for the apical Cl⁻/HCO₃⁻ exchange activity (36, 37, 45, 55). Studies of recombinant DRA and PAT-1 indicate that these anion exchangers conform to the proposed paradigm in that their expression levels change in parallel with CFTR expression (22). In addition to Cl⁻/HCO₃⁻ exchange, Russo et al. (38) have recently reported reduced paracellular Cl⁻ absorption across the small intestine of CF patients, a finding that is also consistent with the hypothesis that absorptive processes are reduced in the secretory-deficient intestine. In contrast to reduced intestinal secretion, hypersecretory states may also show a parallel relationship with the activity of absorptive proteins. In a murine model of osmotic overload diarrhea, both DRA and NHE3 mRNA expression were significantly increased (47). Thus reduced electrolyte and water secretion is compensated by decreased absorption, whereas increased electrolyte and water secretion is compensated by increased absorption.

Studies of the NKCC1(–) intestine demonstrate that stable disruption of other proteins involved in the Cl⁻ secretory pathway also results in parallel reduction in NHE3-mediated Na⁺ absorption. In this case, loss of the principal Cl⁻ uptake protein at the basolateral membrane yielded a reduction in Na⁺ absorption, but unlike the CFTR-deficient intestine, Western blot analysis did not indicate a reduction in NHE3 protein expression. This result was unanticipated, because initial studies of NKCC1(–) small intestine showed reduction in the mRNA expression of NHE3 compared with WT (16). CFTR expression in the NKCC1(–) intestine was also unchanged, thus supporting the concept of a parallel relationship between CFTR and NHE3 expression. As mentioned previously, stable redistributions of NHE3 between endomembranes and the plasmalemma or changes in the activity/expression of NHERF scaffolding proteins (57) could account for both WT levels of protein expression and a reduction in Na⁺ transport activity (56). Alternatively, alterations in actin cytoskeletal organization can have profound inhibitory effects on NHE3 activity without altering the number of exchangers in the plasma membrane (29). The latter possibility deserves consideration because NKCC1 provides a major mechanism of cell volume regulation (23, 24, 32), and in the NKCC1(–) intestine, indexes of epithelial cell volume are significantly reduced compared with WT intestine [NKCC1(–) villous cell area = 145.1 ± 2.4 μm² vs. WT villous cell area = 170.8 ± 3.6 μm²; n = 62 and 56 cells from 3 mice, respectively; P > 0.05, from Ref. 20]. As in other epithelia (35, 46), reduced cell volume in villous epithelium strongly inhibits activity of NHE3 (20). In addition to interactions between cytoskeletal elements and NHE3, other mechanisms causing reduced Na⁺ absorption in the NKCC1(–) intestine might include alterations in the expression/activity of the Na⁺-K⁺-ATPase or differences in intracellular levels of cAMP/cGMP. Thus additional studies will be necessary to investigate mechanisms other than changes in protein expression by which Na⁺ absorption is reduced in the secretory-deficient intestine.

The observations of the present study suggest a homeostatic principle whereby the processes involved in NaCl absorption and secretion across the intestinal epithelium are regulated in parallel. At the molecular level, the interplay between CFTR and NHE3 are most obvious. In the absence of NHE3 protein, CFTR expression is reduced and the anion secretory capacity...
of the intestine is reduced. In the absence of CFTR activity, NHE3 protein expression is significantly reduced, which correlates with a specific decrease in electroneutral Na\(^+\)/H\(^+\) absorption. The specific process of gene regulation, the developmental stage at which regulation occurs, and the influence of pathological/inflammatory factors on transporter expression in the intestine of these mouse models remain to be determined.

It also should be noted here that protein expression was measured using isolated epithelium from the entire small intestine, whereas for comparison to past and present studies, transport function was measured from defined regions of the small intestine. Thus it is possible that regional differences in

![Image](http://ajpgi.physiology.org/DownloadedFrom/10.220.33.4/930/2017/fig6.png)

Fig. 6. Anti-NHE3 immunoblot analysis of epithelial membranes from WT, CFTR\((-\))\), ΔF508/ΔF508 CFTR, and NKCC1\((-\)) small intestine. A: immunoblot using an anti-NHE3 antibody with intestinal epithelial cell membranes from gender-matched littermate WT (CFTR\(+\/+\)) and CFTR\((-\)) (CFTR\(--\)) small intestine. The immunoblot shown was performed on pooled samples from 6 WT and CFTR\((-\)) pairs (immunoblots performed on pooled samples were repeated twice and yielded similar results). Band intensity values were corrected using a calibration curve composed of serial dilutions of an NHE3-enriched sample (see MATERIALS AND METHODS). The pooled CFTR\((-\)) samples exhibited a 34\% reduction in NHE3 protein expression compared with WT. Corrected band intensities for individual mouse immunoblots also yielded an average 34\% reduction in NHE3 protein expression [WT = 29 ± 4 vs. CFTR\((-\)) = 20 ± 2 A.U., \(n = 6\)]. B: immunoblot using an anti-NHE3 antibody with intestinal epithelial cell membranes from gender-matched littermate WT (\(+\/+\)) and ΔF508/ΔF508 CFTR (Δ/Δ) murine small intestine. Pooled intestinal epithelial cell membranes from 20 WT and 20 ΔF508/ΔF508 CFTR mice were used (immunoblots performed on pooled samples were repeated twice and yielded similar results). The ΔF508/ΔF508 CFTR mice exhibited a 26\% reduction in NHE3 protein expression compared with WT.

Interestingly, an additional band at ~75 kDa was stained, but this band was also present in some of the NHE3\((\)+) samples, indicating a different protein than NHE3. The quantity of the ~75-kDa band also did not differ between the WT and NKCC1\((\)+) intestinal samples (~5\%). D: immunoblot using an anti-CFTR antibody with intestinal epithelial cell membranes from gender-matched littermate WT and NKCC1\((-\)) murine small intestine. The immunoblot shown was performed on pooled samples from 6 WT and NKCC1\((-\)) mouse pairs (immunoblots performed on pooled samples were repeated twice and yielded similar results). The pooled NKCC1\((-\)) samples did not differ in CFTR protein expression compared with WT. Corrected band intensities for individual mouse immunoblots exhibited an increase of 12\% in CFTR protein expression [WT = 33 ± 2 vs. CFTR\((-\)) = 37 ± 5 A.U., \(n = 6\)].
transport function along the small intestine may yield results that vary with the present findings. Finally, studies of the NKCC1(−) intestine and changes in paracellular Cl− permeability in the CF intestine suggest that our proposed homeostatic principle extends to processes of regulation that are not directly dependent on changes in transporter expression. Studies within this framework will be necessary to determine expression-independent mechanisms that provide beneficial adjustments to imbalance between the processes of secretion and absorption. Together, these factors contribute to the paradigm that chronic perturbation of electrolyte and water absorption or secretion across the intestine is compensated by parallel changes in the opposing transport pathway, thereby moderating enterosystemic fluid imbalance in disease.

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PARALLEL CHANGES IN ABSORPTION AND SECRETION


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