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Differential regulation of cholera toxin-inhibited Na-H exchange isoforms by butyrate in rat ileum

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Subramanya SB, Rajendran VM, Srinivasan P, Nanda Kumar NS, Ramakrishna BS, Binder HJ. Differential regulation of cholera toxin-inhibited Na-H exchange isoforms by butyrate in rat ileum. *Am J Physiol Gastrointest Liver Physiol* 293: G857–G863, 2007. First published August 9, 2007; doi:10.1152/ajpgi.00462.2006.—Electroneutral Na absorption occurs in the intestine via sodium-hydrogen exchanger (NHE) isoforms NHE2 and NHE3. Bicarbonate and butyrate both stimulate electroneutral Na absorption through NHE. Bicarbonate- but not butyrate-dependent Na absorption is inhibited by cholera toxin (CT). Long-term exposure to butyrate also influences expression of apical membrane proteins in epithelial cells. These studies investigated the effects of short- and long-term in vivo exposure to butyrate on apical membrane NHE and mRNA, protein expression, and activity in rat ileal epithelium that had been exposed to CT. Ileal loops were exposed to CT in vivo for 5 h and apical membrane vesicles were isolated. ^{22}Na uptake was measured by using the inhibitor HOE694 to identify NHE2 and NHE3 activity, and Western blot analyses were performed. CT reduced total NHE activity by 70% in apical membrane vesicles with inhibition of both NHE2 and NHE3. Reduced NHE3 activity and protein expression remained low following removal of CT but increased to control values following incubation of the ileal loop with butyrate for 2 h. In parallel there was a 40% decrease in CT-induced increase in cAMP content. In contrast, NHE2 activity partially increased following removal of CT and was further increased to control levels by butyrate. NHE2 protein expression did not parallel its activity. Neither NHE2 nor NHE3 mRNA content were affected by CT or butyrate. These results indicate that CT has varying effects on the two apical NHE isoforms, inhibiting NHE2 activity without altering its protein expression and reducing both NHE3 activity and protein expression. Butyrate restores both CT-inhibited NHE2 and NHE3 activities to normal levels but via different mechanisms.

ileal mucosa; apical membrane vesicles; ^{22}Na uptake; NHE2 and NHE3 isoforms; protein expression; differential regulation

SHORT CHAIN FATTY ACIDS (SCFA), acetate, propionate, and butyrate, are absorbed in the colon and ileum. In the colon, SCFA stimulate Na and fluid absorption secondary to enhancement of electroneutral Na-Cl absorption (3). The model of SCFA stimulation of Na-Cl absorption is primarily based on in vitro studies using apical membrane vesicles and ion fluxes across isolated colonic mucosa that include an apical membrane mechanism of SCFA uptake (i.e., SCFA-HCO_3 exchange) coupled to parallel Na-H and Cl-butyrate exchanges (3, 23). Although the mechanism of SCFA absorption is controversial with evidence for both apical membrane SCFA-HCO_3 exchange and nonionic diffusion (29), both of these apical membrane processes for SCFA uptake will result in a relative intracellular acidosis, which is required for maximal function of Na-H exchange that is coupled to Cl-SCFA exchange (23).

Increases in mucosal cAMP inhibit intestinal HCO_3-dependent Na-Cl absorption as a result of its inhibition of apical membrane Na-H exchange (2, 21). In contrast to these observations with HCO_3-dependent Na-Cl absorption, in vitro and in vivo experiments in large intestine have established that cAMP does not alter SCFA-dependent Na-Cl absorption (2, 24). To explore the explanation for this apparent paradox of the failure of cAMP to inhibit SCFA-dependent Na-Cl absorption, prior studies addressed the possibility that the two different colonic apical membrane NHE isoforms, NHE2 and NHE3, had both varying responses to cAMP and different regulation of HCO_3-dependent and butyrate-dependent Na-Cl absorption (14, 32). These Na flux studies, in which dibutyryl cAMP (dbcAMP) was added directly to isolated colonic mucosa, made three principal observations: *1*) HCO_3-dependent Na absorption was linked only to NHE3 isoform and not to NHE2 isoform; *2*) butyrate-dependent Na absorption, in contrast, was associated with both NHE3 and NHE2 isoforms; and *3*) dbcAMP inhibited NHE3 isoform, as expected, but increased NHE2 isoform (13). As a result, these observations are consistent with the central role of Na-H exchange with SCFA-dependent Na absorption and the apparent failure of cAMP to inhibit SCFA-dependent Na-Cl absorption.

Whereas the earlier studies investigated the effects of dbcAMP and butyrate on SCFA-dependent Na transport processes in vitro, prolonged in vivo exposure to butyrate may have effects on these transport processes that will likely be relevant to clinical situations in which such changes can be therapeutically exploited. The present experiments were designed to explore further the role of butyrate in the regulation of Na-H exchange by cAMP by performing experiments in which endogenously increased mucosal cAMP levels were maintained over several hours, in contrast to the experimental design of the prior studies in which dbcAMP was acutely added directly to intestinal mucosa in vitro (14). In these present experiments we observed that, as expected, cholera toxin (CT) increased mucosal cAMP levels and decreased H concentration gradient-dependent ^{22}Na uptake but that subse-

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quent exposure of intact intestinal mucosa to butyrate in vivo reduced mucosal cAMP levels and increased NHE activity.

MATERIALS AND METHODS

Sprague-Dawley rats weighing 200–250 g were used in all the experiments. These experiments were approved by the Yale University Animal Care and Use Committee.

In vivo incubation of CT in ileal loops. Rats fasted for 24 h were anesthetized with pentobarbital sodium (40 mg/kg body wt). At laparotomy a 10-cm ileal loop was constructed 5 cm proximal to the ileocecal junction. Ileal loops were flushed with warm HCO_3-Ringer solution (in mM: 140 Na, 5.2 K, 1.2 Mg, 1.2 Ca, 119.8 Cl, 25 HCO_3, 2.4 PO_4, and 1.2 HPO_4) (37°C) and then instilled with either 2 ml Ringer containing 100 μg CT (experimental) or 2 ml Ringer alone (control). The abdomen was then closed, and the anesthetized rats were maintained under heating lamp to maintain the body temperature at 37°C. In additional experiments, following the 5-h CT incubation an additional 2 h incubation was performed with 2 ml of either butyrate-Ringer solution (in mM: 140 Na, 5.2 K, 1.2 Mg, 1.2 Ca, 40 butyrate, 104.8 Cl, 2.4 PO_4, and 1.2 HPO_4) or isethionate-Ringer solution (in mM: 140 Na, 5.2 K, 1.2 Mg, 1.2 Ca, 40 isethionate, 104.8 Cl, 2.4 PO_4, and 1.2 HPO_4) instilled in ileal loops that had previously been incubated with CT. Following these incubations, the ileal loop contents were aspirated and the lengths were recorded. Net fluid movement was determined by calculating the difference between the initially instilled fluid volume and the fluid volume collected following the experiment. The rate of fluid movement was expressed as microliters per 5 h per centimeter. Positive and negative fluid movement represents net absorption and net secretion, respectively.

In additional control studies, ileal loops (not previously exposed to either CT or Ringer) were instilled with either butyrate-Ringer or isethionate-Ringer solutions and were incubated for 2 h. Ringer solutions containing HCO_3 were gassed with 95% O_2-5% CO_2, whereas Ringer solutions containing butyrate or isethionate were gassed with 100% O_2. All solutions were adjusted to pH 7.4.

AMV. Mucosa was scraped from both control and experimental ileal loops, and apical membrane vesicle preparation (AMV) was isolated by the divalent cation chelation method, as described previously by Stieger et al. (30). AMVs suspended in appropriate volume of vesicle buffer (in mM: 50 Mes-Tris, 150 K-gluconate, pH 5.5) were stored at −80°C until use. AMVs were used for both uptake studies and Western blot analyses. Protein assays were performed by the method of Lowry et al. (18).

Uptake studies. Outward H concentration gradient-dependent ^{22}Na (0.1 mCi/mmol, Amersham, Arlington Heights, IL) uptake, as a measure of Na-H exchange (NHE) activity, was performed by rapid filtration techniques, as described previously (30). AMVs were thawed at room temperature and passed through a 26-gauge needle. Uptake was measured for 6 s by diluting 10 μl of vesicles into 90 μl of uptake medium (in mM: 50 HEPES-Tris, pH 7.4, 150 K-gluconate, 1 μM HOE694, 0.1 Na-gluconate, and trace of ^{22}Na). Uptake was arrested by the addition of 1 ml of ice-cold stop solution (in mM: 50 HEPES-Tris, 150 K-gluconate, pH 7.4) and the AMVs were collected on 0.45 μM filter (HAWP Millipore) that was under constant vacuum were solubilized in liquid scintillation fluid. ^{22}Na activity was determined by liquid scintillation spectroscopy.

Apical membrane-specific NHE activities were measured in the presence of either 1 μM or 50 μM HOE694 (kindly provided by Dr. Hans J. Lang, Aventis, Frankfurt, Germany), which inhibits different NHE isoforms in a concentration-dependent manner (7, 13, 34). NHE activity measured in the presence of 1 μM HOE694 represents total apical membrane NHE activity as 1 μM HOE694 inhibits basolateral membrane NHE-1 isoform (34); NHE activity in the presence of 50 μM HOE694 represents the NHE3 fraction, whereas the NHE2 fraction was derived by subtracting NHE3 activity from total NHE activity.

RNA isolation and quantitative PCR. Total RNA was extracted using Trizol reagent (Sigma) and converted to cDNA in a final volume of 20 μl containing 0.5 mM of each nucleotide triphosphate, 40 units of RNAase inhibitor (Ambion), 50–100 ng of random hexamers (Amersham Pharmacia), 200 units of Moloney Murine Leukemia virus RT (Finnzymes), and 1 μg of the extracted RNA and incubated at 42°C for 1 h. Diluted cDNA sample (1 μl) was amplified in a thermal cycler (Chromo4, Bio-Rad) in 20 μl of 1× PCR buffer containing 200 μM of each nucleotide triphosphate (Finnzymes), 2.5 U Hot Gold Star polymerase (Eurogentec), 250 nM forward and reverse primers (IDT), and 1:50,000 diluted SYBRgreen dye (Amersham). Thermal cycling was carried out with initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing (at 59°C for NHE2 and NHE3 and at 56°C for GAPDH), and extension at 72°C for 30 s, then followed by measurement of fluorescence. Product specificity was confirmed by the presence of a single peak in the melting curve analysis. The primer sequences used for these studies are listed in Table 1. The concentration of mRNA for NHE2 and NHE3 was expressed relative to that of the house keeping gene GAPDH by the $2^{-\Delta\Delta}$ C_T method (17).

Western blot. Twenty-five micrograms of apical membrane protein electrophoresed on SDS-PAGE (7.5%) transferred to nitrocellulose membrane was blocked with 5% Blotto overnight at 4°C. The blot was exposed to primary antibody (NHE3 1:2,000; NHE2 1:2,000; β-actin 1:5,000) at room temperature for 1 h. After the primary antibody was washed off, the blots exposed to NHE3 and NHE2 antibodies were exposed to horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit, respectively. For internal control, blots were also probed with β-actin (Sigma, St. Louis, MO). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Bioscience, Piscataway, NJ). Monoclonal NHE3 antibody (1) was generously provided by Dr. Daniel Beimersderfer (Yale University, New Haven, CT). Polyclonal NHE2 antibody (12) was a generous gift from Dr. Mark Donowitz (Johns Hopkins School of Medicine, Baltimore, MD). Secondary antibodies and anti-β-actin were obtained from Sigma. Protein expressions were quantitated by use of personal densitometer SI ImageQuant software.

cAMP assay. Cellular cAMP contents of scraped ileal mucosa was assayed using a nonacetylation enzyme immunoassay kit (Amersham Biosciences, cat. no. RPN225). Mucosal scrapings were obtained from ileal loops that were instilled with Ringer and CT-containing Ringer solutions for 5 h, and isethionate-Ringer and butyrate-Ringer solutions for 2 h following the 5-h experiment with CT.

Statistics. Unless otherwise indicated in the legends, all the results presented represent means ± SE from 4 different AMV preparations. Unpaired Student's *t*-test was performed to test significance of differences between groups. Two-tailed $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS software. In the mRNA studies statistical analyses were performed by Mann-Whitney test.

RESULTS

Effect of CT on fluid secretion and mucosal cAMP level in ileum. Studies were designed to maintain elevated mucosal cAMP levels over prolonged periods that are generally required to affect transport properties at translational and/or transcriptional levels. In these initial studies rat ileal loops

Table 1. *Primer sequences used in RT-PCR studies*

Gene	Forward	Reverse
NHE2	CTGAGCAGCGTGAGTGAGAC	ggcaatgatgaactggtcct
NHE3	CTCTGGGGCAGGAATTGATA	GAAGTTGCTGGGAGAGTTGG
GAPDH	agatggtgaaggtcggtgtc	attgaacttgccgtgggtag

were exposed to CT-Ringer for 5 h. Net fluid absorption was present in the control ileal loops. As predicted, there was both a fourfold increase in mucosal cAMP content ($P < 0.001$) (Fig. 1) and the induction of net fluid secretion in ileal loops exposed to CT for 5 h compared with ileal loops instilled with normal Ringer for 5 h (70.0 ± 5.5 vs. −113.0 ± 11.4 μl/5 h; $P < 0.001$). These observations establish that this CT-Ringer-exposed ileal loop could be used to assess the long-term effect of increased cAMP exposure on ileal electrolyte transport.

Effect of CT on NHE activities in AMV. Experiments were performed to identify whether CT-Ringer-induced fluid secretion is accompanied by concomitant inhibition of Na absorption (NHE activities) in apical membranes of ileum. As shown in Fig. 2*A*, total NHE activity was reduced by 70% in AMV from ileum instilled with CT-Ringer compared with that present in AMV from ileum instilled with normal Ringer. Additional experiments were performed to identify whether CT inhibited specific NHE isoform function, because both NHE2 and NHE3 isoforms are present in AMV from human, rabbit, and rat ileum (12, 33). In these studies NHE2 and NHE3 isoform-specific activities were distinguished by use of HOE694, a NHE inhibitor that alters specific NHE isoform in a concentration-dependent manner (7). As shown in Fig. 2, *B* and *C*, ~30 and 70% of the total NHE activities were contributed by NHE2 and NHE3 isoform-specific functions in normal ileal AMV, respectively. This observation is consistent with observations in apical membranes of rabbit and rat ileum and in rat colon (6, 33). Parallel to total NHE activities, both NHE2 and NHE3 activities were also inhibited in AMV from CT-Ringer incubated ileum (Fig. 2, *B* and *C*). These observations indicate that increased mucosal cAMP inhibits both NHE2 and NHE3 isoform-specific activities.

Effect of butyrate on NHE activities in AMV. Butyrate has been shown to block cAMP inhibition of electroneutral Na absorption in rat colon (14). Therefore, studies were designed to identify whether butyrate could reverse CT-Ringer-inhibited NHE activities. In these studies NHE activities were measured in AMV isolated from ileal loops that were incubated for 2 h with either 40 mM butyrate-Ringer or 40 mM isethionate-Ringer following an initial 5 h CT-Ringer instillation. As shown in Fig. 2, incubation with isethionate, following removal of luminal CT, resulted in a slight increase in total NHE activity without an increase in NHE3 activity. In contrast, NHE2 activity in the presence of isethionate (i.e., following withdrawal of CT) was significantly increased. Incubation with butyrate (again following withdrawal of CT) resulted in substantially increased total NHE activity; NHE activity returned to values comparable to controls that were never exposed to CT. Increase in total NHE activity after butyrate incubation

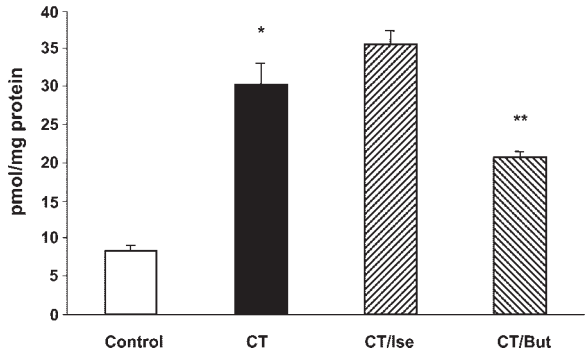

Fig. 1. Effect of butyrate on cholera toxin-elevated cAMP level in ileal mucosal cells. cAMP content was determined in mucosal cells scraped from ileum instilled with either Ringer alone (Control), Ringer containing cholera toxin (CT) for 5 h, CT-Ringer followed by 2 h 40 mM isethionate (CT/Ise) or CT-Ringer followed by 2 h 40 mM butyrate (CT/But). *$P < 0.001$ compared with control. **$P < 0.001$ compared with CT/Ise.

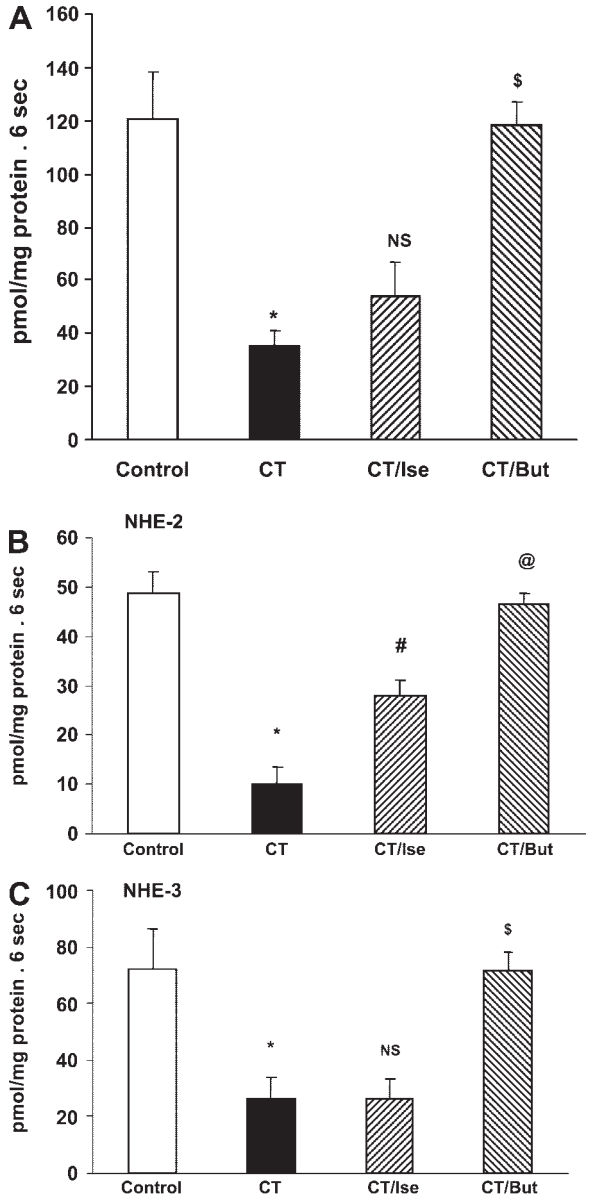

Fig. 2. Effect of butyrate on cholera toxin-inhibited sodium-hydrogen exchanger (NHE) activities in ileal apical membrane vesicles. *A*: total NHE activity, in apical membrane vesicle preparations (AMV) isolated from ileal loops instilled with either Ringer alone for 5 h, Ringer containing 100 μg cholera toxin for 5 h, 5 h CT-Ringer followed by 2 h with 40 mM isethionate-Ringer, or 5 h CT-Ringer followed by 2 h with 40 mM butyrate-Ringer. NHE activities were measured in the presence and absence of 50 μM HOE694. HOE-sensitive NHE activity represents the NHE2 fraction (*B*), and HOE-insensitive component represents the NHE3 fraction (*C*). Total NHE and NHE isoform-specific activities were calculated, as described in MATERIALS AND METHODS. *$P < 0.001$ compared with control. #$P < 0.001$ compared with CT. $$P < 0.001$ compared with CT/Ise. @$P < 0.001$ compared with CT/Ise. NS, not significant, compared with CT.

was due to increases in both NHE3 and NHE2 activities (Fig. 2, *B* and *C*) which were essentially similar in magnitude to that in control loops never exposed to CT. Thus 2-h incubation with butyrate following CT exposure completely reversed the inhibitory effect of CT (i.e., cAMP) on NHE activity. These observations suggest that the inhibition of NHE2 activity by CT was rapidly reversible and transient, since this activity rapidly increased in the presence of isethionate-containing Ringer. On the other hand, removal of CT did not modify NHE3 activity, whereas the presence of butyrate resulted in a return in NHE3 activity to normal following CT exposure. These observations suggest that the modes of CT inhibition of NHE2 and NHE3 activities differ.

To establish whether the reversal of NHE activities following CT withdrawal and butyrate exposure was immediate or required a period of time, the following experiment was performed. NHE activity was first inhibited by incubation for 5 h with CT-Ringer and then exposed to butyrate-Ringer or isethionate-Ringer for 10 min, following which AMVs were prepared from the ileal loops and used for transport studies. The removal of CT in the presence of isethionate (9.9 ± 4.2 vs. 29.2 ± 6.3 pmol/mg protein; 6 s; $P < 0.001$) or butyrate (9.9 ± 4.2 vs. 30.2 ± 5.4 pmol/mg protein; 6 s; $P < 0.001$) resulted in a threefold increase in NHE2 activity, which is identical to that observed in the presence of isethionate during a 2-h incubation (Fig. 2*B*). In contrast, NHE3 activity was not altered by the removal of CT in the presence of either isethionate or butyrate (data not shown). These observations establish that the effect of butyrate on both NHE2 and NHE3 activities requires more than 10 min.

To establish whether butyrate increases only CT-inhibitable NHE activity or whether it also activates basal NHE activity, total NHE activity was measured in AMV isolated from normal ileal loops that were instilled with either butyrate-Ringer or isethionate-Ringer for 2 h. Butyrate did not alter total NHE activities in AMV of normal ileal loops compared with isethionate group (104.2 ± 12.4 vs. 101.4 ± 13.5 pmol/mg protein; 6 s). These results indicate that butyrate reversed CT-inhibition of NHE activity but did not augment basal NHE activity.

Effect of CT and butyrate on NHE2- and NHE3-specific protein expression in AMV. To establish whether CT inhibition of NHE2 and NHE3 activity and their reversal by CT removal of butyrate occurred as a result of functional changes in these transporters or whether this was due to deletion and insertion of membrane proteins, Western blot analyses were performed with apical membranes using NHE3 and NHE2 isoform-specific antibodies. As shown in Fig. 3, NHE3 protein expression was decreased approximately by 60% in apical membranes from ileal loops that were instilled with CT-Ringer. NHE3 protein expression was not altered in apical membranes from ileal loops exposed to isethionate-Ringer following the removal of CT. NHE3 protein expression increased by threefold in apical membranes from ileal loops that were exposed to butyrate-Ringer following CT-Ringer (Fig. 3). In contrast to reduced NHE3 protein expression, CT-Ringer instillation did not reduce, but modestly increased NHE2 protein expression (Fig. 4). Following the removal of CT, butyrate, but not isethionate, enhanced NHE2 protein expression in CT-Ringer-exposed ileum (Fig. 4). Neither CT, isethionate, nor butyrate affected the level of expression of the housekeeping protein β-actin. These observations establish that CT reduced NHE2 and NHE3 activities by different mechanisms. CT reduced NHE3 activity by decreasing NHE3 protein expression in apical membranes, whereas butyrate reversed CT-reduced NHE activity by enhancing NHE3 protein expression in apical membranes. In contrast, CT reduced NHE2 activity without altering NHE2 protein expression.

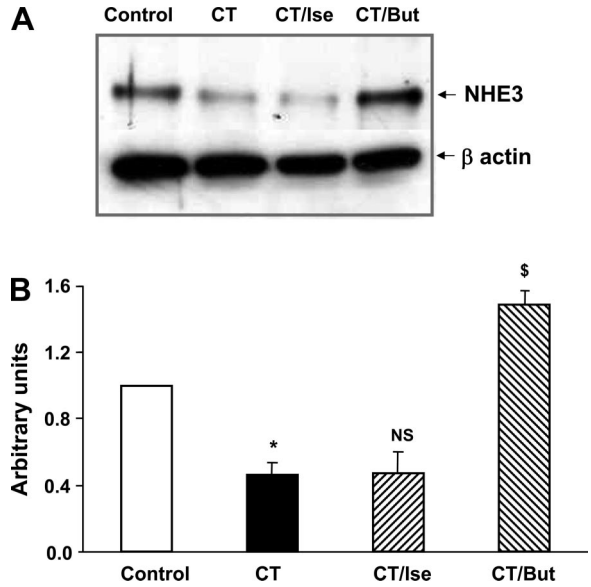

Fig. 3. Effect of butyrate on CT-reduced NHE3 isoform-specific protein expression in ileal apical membranes. *A*: Western blot analyses of apical membranes isolated from ileum incubated with either Ringer alone, Ringer containing cholera toxin, CT-Ringer followed by 2 h isethionate, or CT-Ringer followed by 2 h butyrate. Immunoblots were performed using NHE3 isoform-specific monoclonal antibody (1). Western blot was also performed using β-actin antibody as an internal control. *B*: relative expression of NHE3-specific protein in apical membrane of Control, CT, CT/Ise, and CT/But were quantitated by densitometry from 3 different membrane preparations. $^*P < 0.005$ compared with control. $\$P < 0.001$ compared with CT.

Effect of CT and butyrate on NHE2 and NHE3 mRNA. NHE2 and NHE3 mRNA levels were determined using quantitative real-time PCR and expressed relative to that of GAPDH mRNA. Minor changes were present between the groups, but none were significantly different from their corresponding control.

Effect of butyrate on cAMP levels. The above results establish that CT inhibited NHE3 activity and protein expression, which was reversed by in vivo exposure to butyrate. Experiments were designed to establish whether the effects produced by butyrate were mediated by changes in mucosal cAMP content. Figure 1 presents the results of these experiments in which CT-increased mucosal cAMP content following incubation with butyrate-Ringer for 2 h resulted in an approximate 40% reduction in mucosal cAMP levels. In contrast, incubation with isethionate-Ringer did not affect mucosal cAMP levels.

DISCUSSION

The present experiments explored the apparent paradox that the model of butyrate-dependent Na absorption includes an apical membrane Na-H exchange; that cAMP is known to inhibit NHE3 isoform, an apical membrane Na-H exchange; but that cAMP does not inhibit butyrate-dependent Na absorption (2, 23). To address this important issue, experiments were designed that resulted in prolonged increases in mucosal cAMP

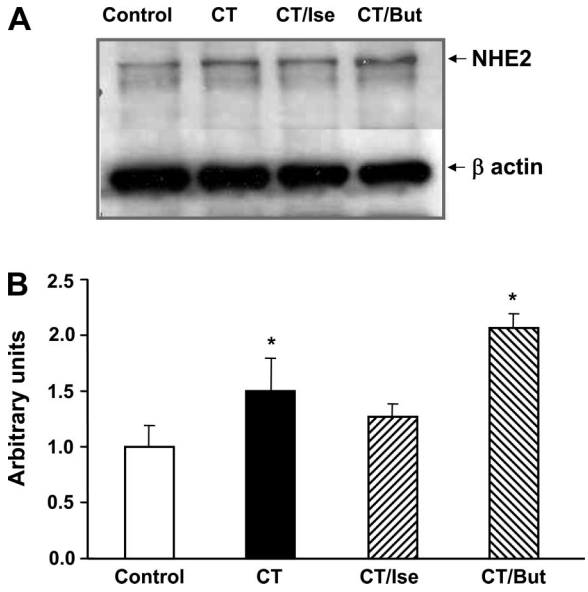

Fig. 4. Effect of butyrate on NHE2 isoform-specific protein expression in CT-treated ileal apical membranes. *A*: Western blot analyses of apical membranes isolated from ileum incubated with either Ringer alone, Ringer containing cholera toxin, CT-Ringer followed by 2 h isethionate, or CT-Ringer followed by 2 h butyrate. Immunoblots were performed using NHE2 isoform-specific polyclonal antibody (12). Western blot was also performed using β-actin antibody as an internal control. *B*: relative expression of NHE2 specific protein in apical membrane of Control, CT, CT/Ise, and CT/But were quantitated by densitometry from 3 different membrane preparations (12). $*P < 0.001$ compared with control.

in rat ileal mucosa following the instillation of CT. As expected in this in vivo model, enhanced levels of mucosal cAMP for up to 5 h were demonstrated together with reduced NHE2 and NHE3 activities, as determined by ^{22}Na uptake by AMV (Fig. 2).

The novel observation of these present studies was that these two central findings, i.e., the increase in mucosal cAMP and the reduction in NHE activities, were partially or completely reversed by exposing the ileal mucosa in vivo to butyrate for 2 h (Figs. 1 and 2). Control experiments established that neither in vivo incubation of ileal mucosa with butyrate for 10 min nor in vitro incubation of AMV with butyrate for 2 h was sufficient to induce these changes in NHE activities (unpublished observations). The decrease in mucosal cAMP levels by butyrate could be due to either activation of phosphodiesterases or inhibition of adenylate cyclase by butyrate. Evidence for both of these possible mechanisms has been reported (26, 35). However, a definitive molecular understanding how butyrate reverses or prevents the effects of cAMP on NHE has not as yet been established.

Butyrate has multiple effects on intestinal epithelial function. Butyrate affects both cell differentiation and ion transport (5, 8, 14, 15). First, butyrate inhibits cAMP- and cGMP-stimulated Cl secretion but not Ca-mediated Cl secretion (26). Second, butyrate also inhibits both Cl-dependent HCO_3 secretion and cAMP-stimulated HCO_3 secretion (31). Third, NHE expression is upregulated in intestinal epithelial C2bbe cells by butyrate when incubated for 48 h (19). Fourth, butyrate enhances fluid absorption as a result of stimulating electroneutral Na-Cl absorption (1). Fifth, butyrate inhibits histone deacylation, which may be associated with its effect to increase cell differentiation.

It would be important to compare the present results that were performed in vivo with prior in vitro experiments that had also been designed to explain the failure of cAMP to inhibit butyrate-dependent Na absorption (13) as well as the two initial observations that cAMP did not affect SCFA-dependent Na and fluid absorption (24). In the previous experiments both dbcAMP and butyrate were added to intact colonic mucosa in vitro for no more than 20 min prior to the determination of Na transport. This experimental design parallels the demonstration that the acute addition of either dbcAMP or theophylline did not alter butyrate-dependent Na absorption in vitro. In contrast, the present experiments were performed in vivo in which mucosal cAMP levels were elevated secondary to CT for several hours prior to the transport studies.

The clinical application of this paradox that cAMP does not inhibit butyrate-dependent Na absorption has been the development of a modified oral rehydration solution (ORS) by the addition of amylase-resistant starch (RS), i.e., starch that is relatively resistant to amylase digestion, to ORS (25). The effectiveness of WHO-ORS, an isoosmolar glucose-electrolyte solution, is based on the physiological principles that absorptive and secretory processes are separate and distinct, that stimulation of Cl secretion does not affect glucose-stimulated Na absorption because cAMP that induces Cl secretion does not alter glucose-stimulated Na absorption, and that stimulation of glucose-stimulated Na absorption does not alter activated Cl secretion (10). As a consequence, WHO-ORS, which enhances fluid and Na absorption in the small intestine, has dramatic effects to correct the dehydration and metabolic acidosis associated with severe diarrhea especially in children but does not result in substantial reduction in diarrhea (9, 11). The initial proposal to employ RS-ORS was based on the expectation that RS would result in an increase in the delivery of nonabsorbed carbohydrate to the colon (4). As a consequence, there would be an increase in the production of SCFA, which in turn would result in an increase in colonic fluid and Na absorption in the colon. Clinical studies in both adults with acute cholera (25) and children with noncholera diarrhea (20, 22) have confirmed this hypothesis by the demonstration that RS-ORS is significantly better than WHO-ORS in shortening the time to the first formed stool.

These present studies were performed in ileal mucosa because preliminary experiments using rat colon failed to establish a model in which CT consistently induced fluid secretion in vivo. Prior studies have demonstrated that SCFA are absorbed in both small and large intestine by similar mechanisms (27, 28). Under normal conditions SCFA are almost exclusively present in the colon and are the predominant anion in stool because SCFA are not present in the diet and are produced (fermented) by colonic bacteria from nonabsorbed carbohydrate. However, in conditions in which bacteria are found in the terminal ileum as in many healthy residents of the tropics, SCFA may presumably be found in the lumen of the colon.

The effects of CT and cAMP on NHE2 and NHE3 protein levels and functional activities differ. Although cAMP inhibited both NHE3 and NHE2 function equally, only NHE3 protein expression was reduced by increased levels of mucosal cAMP during a 5-h period. This differential regulation of NHE

isoforms by cAMP is consistent with prior studies (14). Furthermore, following the removal of CT and the addition of isethionate and butyrate, NHE3 and NHE2 functional activity and protein expression also differed. NHE3 protein and function parallel each other during the 2-h butyrate experiment, resulting in a significant increase in both NHE3 isoform activity and protein level. On the other hand, NHE2 function increased following CT withdrawal and was further enhanced by butyrate. In addition, NHE2 protein expression was not changed despite the decrease in NHE2 activity. Although a mechanism(s) for these disparate changes is not apparent as yet, these observations establish that CT inhibits both NHE2 and NHE3 activities by different and distinct mechanisms (Fig. 2*B*). This conclusion is consistent with and supplements our earlier demonstration that cAMP inhibits NHE3 but not NHE2 function in rat distal colon (14). In this earlier study we had shown that under basal conditions both HCO_3-dependent and butyrate-dependent Na absorptions are mediated by NHE3 function, whereas in the presence of cAMP butyrate-dependent but not HCO_3-dependent Na absorption is present, and that butyrate-dependent Na absorption is mediated by NHE2 isoform in rat distal colon (14). This differential effect of CT on NHE2 and NHE3 provides a molecular explanation for several clinical and experimental observations.

These present studies establish that the regulation of NHE2 and NHE3 by cAMP differs and are consistent with present concepts regarding the regulation of different NHEs (35). NHE3 protein is present both in apical membranes and in a subapical membrane endosomal compartment (16). Furthermore, NHE3 function is regulated by its trafficking between these two cellular compartments that likely requires phosphorylation. In contrast, NHE2 protein is generally believed to be present solely in the plasma membrane and its regulation is likely a result of changes in its turnover number without evidence of either membrane trafficking or phosphorylation (35). Thus, in the present study, although cAMP inhibited both NHE2 and NHE3 activity (Fig. 2), NHE3 but not NHE2 protein expression was altered by cAMP (Figs. 3 and 4). This observation is consistent with cAMP reducing NHE3 protein recycling to the apical membrane. Because cAMP did not reduce either NHE2 or NHE3 mRNA expression, cAMP's effects on NHE3 activity are at a posttranscriptional level whereas those on NHE2 activity are at a posttranslational level. Since butyrate partially reduced cAMP levels (Fig. 1), it is not known whether all of the increase in NHE2 and NHE3 activity in the presence of butyrate is due to this decrease in cAMP alone or whether butyrate increased NHE activity independent of the change in cAMP.

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