Na-K-2Cl Cotransporter Gene Expression and Function During Enterocyte Differentiation

Modulation of Cl^- Secretory Capacity by Butyrate

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Abstract

The basolateral Na-K-2Cl cotransporter (NKCC1) is a key component of the intestinal crypt cell secretory apparatus. Its fate during the transition to absorptive enterocyte and the potential impact of its altered expression on secretory output have not been addressed. In this report, NKCC1 mRNA was found to be expressed in rat jejunal crypt but not villus cells. Butyrate treatment of intestinal epithelial HT29 cells induced a differentiation pattern that recapitulated the rat intestinal crypt-villus axis, with NKCC1 mRNA levels decreasing in a time- and dose-dependent fashion in parallel with upregulation of apical brush-border markers. Butyrate but not acetate or proprionate decreased basal and cAMP-stimulated bumetanide-sensitive K^+ (^{86}Rb) uptake in both HT29 cells and the Cl^--secreting T84 line. Butyrate markedly decreased transepithelial Cl^- secretion in confluent T84 monolayers without effect on cAMP-regulated apical Cl^- efflux. We conclude that NKCC1 regulation during enterocyte differentiation occurs at the level of gene expression, and that selective downregulation of NKCC1 gene expression and function by butyrate leads to a profound decrease in transepithelial Cl^- secretion. These data emphasize the importance of NKCC1 in determining epithelial secretory capacity and suggest the possibility of modulation of the enterocytic transport phenotype as therapy for diarrheal disorders. (*J. Clin. Invest.* 1998. 101:2072–2079.) Key words: intestinal mucosa • fatty acids • colitis • bumetanide • cystic fibrosis transmembrane conductance regulator

Introduction

Na-K-2Cl cotransport proteins are an integral component of the vectorial transport apparatus of many secretory and absorptive epithelia (1). In the gastrointestinal and respiratory tracts, a basolaterally situated Na-K-2Cl cotransporter mediates salt uptake in concert with a basolateral Na-K ATPase and K^+ channels for the purpose of transepithelial Cl^- secretion, an event ultimately gated by apical plasma membrane Cl^- channels such as the cystic fibrosis transmembrane conductance regulator (CFTR)[1] (2, 3). Epithelial Cl^- secretion accounts for mucosal surface hydration, and several human diseases, ranging from secretory diarrhea to cystic fibrosis, result from its defective regulation. Recent cloning and functional expression studies have identified a family of structurally related cation-chloride cotransporters that uniformly display strict ion interdependence of transport and sensitivity to loop diuretics such as bumetanide (4–7). NKCC1 (BSC2) is the major secretory or basolateral isoform of the Na-K-2Cl cotransporter, and homologues of NKCC1 have been cloned from the shark rectal gland, mouse inner medullary collecting duct, and human colon. Northern blot analysis has shown the highest degree of expression of NKCC1 in the large intestine, stomach, and kidney, but prominent message is also detectable in many other tissues, including the small intestine (5). NKCC2 (BSC1) represents the absorptive isoform that to date has not been identified outside of the kidney (5).

The intestinal epithelium is composed of multiple cell types that originate from pluripotent stem cells located just above the base of the crypts of Lieberkuhn (8). Cl^- secretory function is thought to derive largely from the epithelial cells lining the crypts (9), whereas digestive and absorptive function is a property limited to villus enterocytes (10). This spatial segregation of epithelial function is reflected in a differential pattern of expression of several specific membrane proteins along the crypt-villus axis. For example, RNA in situ hybridization studies indicate high endogenous expression of CFTR in the intestinal crypts but the absence of various apical membrane digestive enzymes and nutrient transporters, whereas the reverse is true for the cells at the villus tips (10, 11). Thus, epithelial cells emerging from the proliferative compartment initially display the capacity for Cl^- secretion, but during the course of epithelial cell migration from crypt to villus tip, these cells are thought to acquire the differentiated functions of mature villus enterocytes while apparently losing the Cl^- secretory phenotype. Details of the molecular mechanisms underlying this transition have been lacking. Research in this area has focused almost exclusively on apical membrane components. For several brush-border markers (e.g., lactase and sucrase/isomaltase), regulation along the crypt-villus axis appears to be largely transcriptional (10, 12, 13). Although the apparent pau-

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1. *Abbreviations used in this paper:* CFTR, cystic fibrosis transmembrane conductance regulator; HPBR, Hepes-phosphate–buffered Ringer's solution; IAP, intestinal alkaline phosphatase; I_{sc}, short-circuit current; SCFA, short-chain fatty acid(s).

city of CFTR mRNA in villus enterocytes suggests that CFTR gene regulation may also occur at the transcriptional level, in vitro models using several available intestinal epithelial cell lines suggest the possibility of translational control as well (14–16). Little is known about the crypt-villus pattern of expression of basolateral membrane proteins such as NKCC1. However, recent functional studies have suggested that NKCC1 represents an important independent regulatory site that may modulate net secretory capacity (1, 17–19).

In this study, we examine NKCC1 gene expression in isolated crypt and villus enterocyte populations of native rat jejunum, and we use butyrate-treated HT29 and T84 human intestinal epithelial cells to examine molecular aspects and functional consequences of altered NKCC1 expression during in vitro enterocyte differentiation. We demonstrate that NKCC1 mRNA expression decreases as a function of differentiation along the crypt-villus axis in vivo and in response to butyrate in both cultured cell lines. Downregulation of NKCC1 expression in vitro occurs independently of changes in CFTR mRNA or cAMP-dependent apical membrane Cl^- efflux, but nonetheless leads to a diminution of transepithelial Cl^- secretion due to decreased basal and cAMP-stimulated Na-K-2Cl cotransport. Some of these results have appeared in abstract form (20, 21).

Methods

Cell culture and buffers. Human colon adenocarcinoma HT29 cells obtained from the American Type Culture Collection (Rockville, MD) were grown in DME supplemented with 10% heat-inactivated FCS and glucose (4.5 g/liter). Cells were grown in a humidified 95% O_2-5% CO_2 incubator at 37°C, fed every 2–3 d, and split 1:5 every 5 d. For molecular studies, experiments were performed at ~ 80% confluence. Transport studies were performed 4–7 d after seeding onto 35-mm Petri dishes which were fed every 2–3 d. Media were changed the day before the experiments. T84 human intestinal epithelial cells obtained from the American Type Culture Collection and Dr. K. Barrett (University of San Diego, La Jolla, CA) were maintained in culture as described previously (18, 22). For experiments, T84 cells were seeded onto 0.33- or 1-cm^2 collagen-coated permeable supports (Costar, Cambridge, MA) and used after confluence and stable transepithelial electrical resistances were achieved, ~ 7–14 d after plating. Uptake and transepithelial transport studies (see below) were carried out at room temperature in a Hepes-phosphate–buffered Ringer's solution (HPBR) (pH 7.40) which contained (mM): 135 NaCl, 5 KCl, 3.33 NaH_2PO_4, 0.83 Na_2HPO_4, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 5 Hepes.

Separation of crypt and villus enterocyte populations. Adult male Sprague-Dawley rats (250–300 g) were maintained on standard food and water. Animals were killed by 40 mg/kg pentobarbital administered intraperitoneally, a laparotomy was performed, and small intestinal tissue was harvested. A previously described technique to separate villus and crypt cell populations was used (23). Intestinal segments were everted to expose the mucosa to a EDTA-saline solution at 37°C. Samples for RNA purification were collected at 30, 60, 90, and 120 min to sequentially obtain cells from the villus, mid-villus, and crypt compartments. Previous Northern blot analyses have confirmed that brush-border enzyme mRNA is largely confined to the first two fractions, whereas mRNA for the Paneth cell marker cryptdin is enhanced in the fourth fraction. Histologic sections have verified the separation technique, showing progressive loss of cells from villus tip to crypt base.

Northern blot analysis. Total RNA was extracted using the guanidium thiocyanate method. Northern blot analysis was then performed by loading 20 μg of RNA in each lane of an agarose-formaldehyde gel, separating through electrophoresis, transferring to nitrocellulose membranes, and baking for 2 h at 80°C. Equal loading of RNA per lane was confirmed by examination of ethidium bromide–stained gels. Complementary cDNA probes were ^{32}P-labeled by the random primer method to a specific activity of $\sim 5 \times 10^8$ cpm/μg DNA. The NKCC1 probe is a 4.3-kb EcoRI/XhoI fragment derived from the human cDNA and was provided by B. Forbush III (Yale University School of Medicine, New Haven, CT) (6). The lactase probe was provided by R. Grand (New England Medical Center, Boston, MA) and is a 1.8-kb EcoRI/PstI fragment derived from the rat cDNA clone (24). An intestinal alkaline phosphatase (IAP) probe was obtained from the American Type Culture Collection and is a 1.9-kb PstI fragment derived from human IAP cDNA (25). The villin probe is a 530-bp fragment from human cDNA and was provided by M. Arpin (Institut Pasteur, Paris, France) (26). The actin probe is a 1.0-kb PstI fragment derived from a mouse β-actin cDNA (27). The cryptdin probe is derived from the mouse cDNA and was provided by A.J. Ouellette (Shriners Burns Institute, Boston, MA) (28). The CFTR probe is an ~ 900-bp EcoRI fragment derived from human cDNA (29). Conditions for hybridization were 5× standard saline citrate/50% (vol/vol) formamide/1% (wt/vol) SDS at 42°C. Washing conditions were 2× standard saline citrate/0.1% SDS at 50°C. Relative changes in mRNA were determined by laser densitometry of autoradiograms and normalized for actin mRNA.

Transepithelial transport and isotopic flux studies. Isotopic flux studies were performed on HT29 cells grown on 35-mm plastic dishes or on T84 monolayers grown on permeable collagen-coated supports, as described previously (18, 30, 31). Na-K-2Cl cotransporter function was determined by ^{86}Rb uptake using monolayers that had been pretreated with butyrate in culture media at the indicated time and concentrations. Control and butyrate-treated monolayers were fed according to identical schedules. Transport studies were always performed in HPBR without butyrate regardless of the pretreatment conditions. Briefly, monolayers were removed from the incubator, the media were washed, and the monolayers were then preequilibrated in HPBR for 20–30 min. Monolayers were then incubated for 10 min in HPBR in the presence or absence of the adenylate cyclase activator forskolin (10 μM) and the NKCC1 inhibitor bumetanide (10 μM). This solution was then aspirated and replaced by an identical solution containing ~ 1.5 μCi/ml ^{86}Rb. For T84 monolayers, only the basolateral buffer contained agonists, inhibitors, and isotopes. After 3 min, uptake was terminated by rapidly washing the dishes or supports four times with an ice-cold solution of 100 mM $MgCl_2$ and 10 mM TRIS (pH 7.4). For HT29 cells, radioactivity was extracted in 1 ml of 0.1 M NaOH. For T84 monolayers, the filters were simply cut from their supports. Total protein was determined on representative monolayers using a bicinchoninic acid assay from Pierce Chemical Co. (Rockford, IL). Uptakes were expressed as nmol K^+/mg protein/min. The activity of the Na-K-2Cl cotransporter was defined by convention as the bumetanide-sensitive component of uptake, a value derived by subtracting the individual values of uptake measured in the absence of bumetanide from the mean value of uptakes measured in the presence of bumetanide, with triplicate monolayers used for each condition.

Activation of apical Cl^- and basolateral K^+ efflux pathways in response to forskolin was measured by a modification of the method of Venglarik et al., using ^{125}I and ^{86}Rb as tracers, respectively, and previously reported techniques (18, 31, 32). Efflux rate constants were expressed as min^{-1}.

Short-circuit current (I_{sc}) has been validated repeatedly as an accurate measure of net Cl^- secretion in the T84 model. I_{sc} was measured in monolayers grown on 0.33-cm^2 permeable supports using a dual voltage current clamp and Ag/AgCl and calomel electrodes interfaced via chopstick KCl-agar bridges by standard techniques.

Materials and analysis. Radioisotopes were obtained from DuPont NEN (Boston, MA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). Data are expressed as mean±SEM. Statistical analysis was performed by Student's paired *t* test and by ANOVA, where appropriate, with a $P < 0.05$ considered significant.

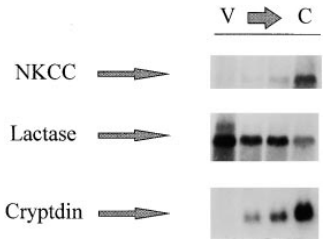

Figure 1. NKCC1 gene expression along the crypt-villus axis. Representative Northern analyses are shown depicting the pattern of NKCC1 mRNA expression along the crypt (*C*)-villus (*V*) axis. The depicted RNA is derived from the 30-, 60-, 90-, and 120-min incubation as cells are extracted from villus tip to crypt base (see Methods). As expected, the Paneth cell specific marker cryptdin mRNA is most abundant in the crypt base fraction, whereas the brush-border enzyme lactase mRNA is highest at the villus tips. 20 μg of total RNA was loaded per lane, with equal loading verified by ethidium bromide staining.

Results

NKCC1 mRNA is differentially expressed in crypt and villus cell populations of rat jejunum. To determine whether mRNA for NKCC1 is variably expressed along the crypt-villus axis of native small intestinal epithelium, serial populations of crypt epithelial cells and villus enterocytes were harvested by established techniques. Crypt-villus separation was verified by the demonstration of high levels of lactase mRNA in the villus enterocyte population (fraction 1) whereas the Paneth cell marker cryptdin was most abundant in the crypt cell population (fraction 4). NKCC1 mRNA was undetectable in cells from the villus tip and midvillus but was expressed abundantly in the crypts (Fig. 1).

Butyrate downregulates NKCC1 mRNA expression in an HT29 model of enterocyte differentiation. HT29 cells are a pluripotent human intestinal epithelial line that has been widely used as a model of enterocyte differentiation (33). In general, HT29 cells do not express typical apical membrane markers of mature enterocytes. Although HT29 cells are unpolarized, they do express mRNA for CFTR, a key cAMP-dependent apical membrane Cl^- channel required for transepithelial Cl^- secretion. However, CFTR protein in HT29 cells remains within Golgi-derived vesicles and is not trafficked to the cell surface (31, 34). Thus, HT29 cells lack a cAMP-stimulated Cl^- efflux pathway. The plasma membrane of HT29 cells displays the basolateral components of epithelial Cl^- secretion, specifically, Na-K ATPase, NKCC1, and several types of K^+ channels (35, 36). By manipulation of culture conditions (e.g., substrate deprivation), HT29 cells may be induced to differentiate into multiple epithelial cell types (33). Sodium butyrate treatment of HT29 cells has been demonstrated previously to induce a differentiation pattern that resembles the transformation of undifferentiated cells into villus enterocytes. Specifically, butyrate-treated HT29 cells withdraw from the cell cycle and begin to express enterocyte-specific apical membrane marker genes such as IAP and villin (13, 37).

In these experiments, butyrate treatment of HT29 cells profoundly decreased mRNA levels for NKCC1 in a time- and dose-dependent fashion. As shown in Fig. 2 *A*, NKCC1 mRNA levels are quite high in untreated HT29 cells; however, there is a dramatic decrease in response to butyrate treatment, primarily occurring between 8 and 24 h. This decrease in NKCC1 expression is in contrast to the induction of the IAP mRNA which also occurred between 8 and 24 h after the addition of butyrate. A dose–response experiment revealed the effects of butyrate on NKCC1 expression were detectable at a concentration of ~ 5 mM (Fig. 2 *B*), similar to that seen with other butyrate effects. Butyrate treatment of HT29 cells induced a differentiation pattern recapitulating that seen along the crypt-villus axis of rat small intestine, as evidenced by the induction of brush-border markers and the disappearance of a key basolateral component of the Cl^- secretory apparatus. Consistent with in vitro differentiation models using this and other cultured intestinal epithelial cell lines (14, 16); however, butyrate treatment did not decrease mRNA levels for CFTR (Fig. 2 *C*), suggesting that butyrate differentially regulates gene expression of apical and basolateral Cl^- transport pathways.

The decrease in NKCC1 mRNA did not require new protein synthesis, as the effects were not abrogated by prior treatment with either cycloheximide or anisomycin (Fig. 3 *A*). We

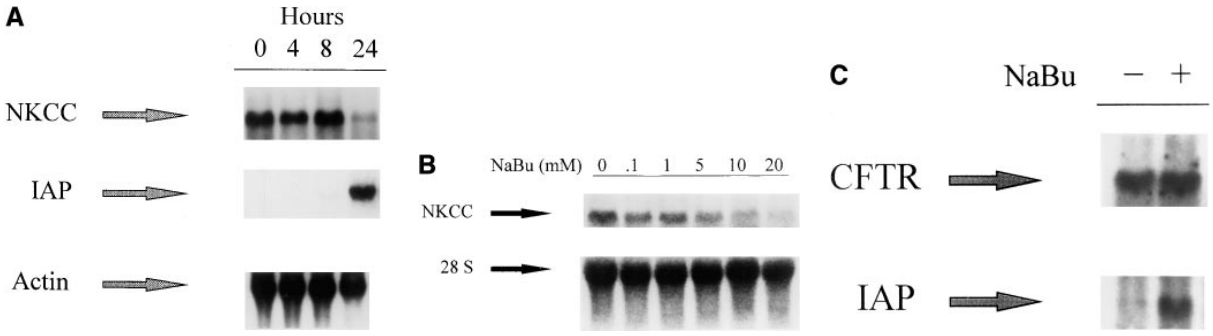

Figure 2. Butyrate downregulates NKCC1 expression in HT29 cells. (*A*) Northern blot analyses were carried out on total RNA derived from HT29 cells treated with 5 mM butyrate for the indicated times (in hours). 20 μg of total RNA was loaded per lane. Hybridizations were performed using radiolabeled cDNA probes specific for NKCC1, IAP, and actin mRNAs. (*B*) Dose-dependent effects of butyrate on NKCC1 gene expression. Representative Northern analyses are shown depicting NKCC1 mRNA expression in HT29 cells as a function of sodium butyrate (*NaBu*) concentration. Cells were treated with 0–20 mM of sodium butyrate for 48 h and total RNA was analyzed. 20 μg of total RNA was loaded per lane, with equal loading verified by ethidium bromide staining and the levels of ribosomal RNA (28S). (*C*) Effect of butyrate on CFTR mRNA expression. Northern blot analyses using total RNA derived from HT29 cells treated for 48 h with 5 mM NaBu. Hybridizations were performed using radiolabeled cDNA probes specific for either CFTR or IAP. Equal loading of RNA per lane was verified by ethidium bromide staining of 28S ribosomal RNA (not shown).

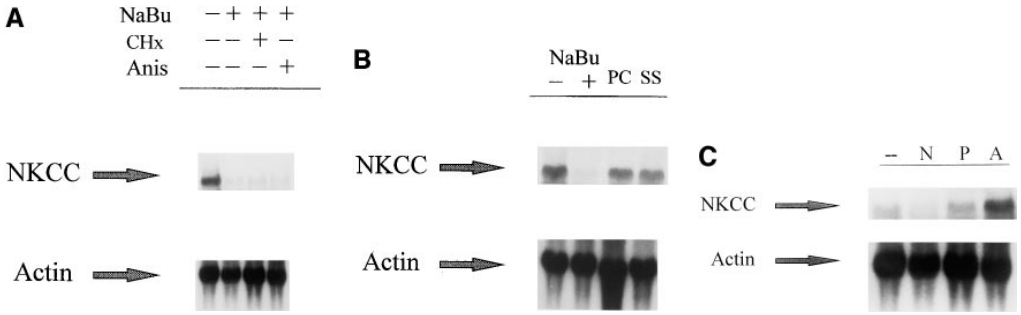

Figure 3. Effects of butyrate on NKCC1 gene expression. The effect of protein synthesis inhibitors, and comparison of effects of serum starvation, postconfluent growth inhibition, and other SCFA is shown. (*A*) Northern analyses were carried out using total RNA derived from HT29 cells treated for 48 h with 5 mM butyrate (*NaBu*) with (+) or without (−) the protein synthesis inhibitors cycloheximide (*CHx*) or anisomycin (*Anis*). In experiments not shown, cycloheximide or anisomycin alone had no effect upon basal NKCC1 mRNA levels. (*B*) Relationship of NKCC1 mRNA levels and cellular growth state. Northern blot analyses were carried out using total RNA derived from HT29 cells treated with (+) or without (−) 5 mM sodium butyrate (*NaBu*), or grown to 30 d postconfluence (*PC*), or in media lacking FCS for 48 h (*SS*). Equal loading of RNA per lane was verified by ethidium bromide staining of the gels, along with probing with the actin control (*bottom*). Note that the dramatic decrease in NKCC1 mRNA levels is not seen under the other two conditions of cell cycle withdrawal (*PC* or *SS*). (*C*) Effects of various SCFA on NKCC1 mRNA levels. Northern blot analyses carried out on 20 μg of total RNA derived from HT29 cells treated with vehicle (−), or 5 mM sodium butyrate (*N*), proprionate (*P*), or acetate (*A*). Hybridizations were performed using radiolabeled cDNA probes specific for NKCC1 and actin mRNAs.

examined the possible role of cellular growth state in NKCC1 gene expression, since butyrate has been shown to cause cell cycle arrest in HT29 cells (13, 37). As shown in Fig. 3 *B*, NKCC1 mRNA levels were unaltered in cells grown to 30 d after confluence or under conditions of serum starvation, indicating that the decrease in NKCC1 expression by butyrate is not due merely to decreased cell growth. Cycloheximide alone caused no change in NKCC1 mRNA levels. The short-chain fatty acids (SCFA) acetate and proprionate in equimolar concentrations did not decrease NKCC1 mRNA levels, demonstrating specificity of the downregulating effect for butyrate (Fig. 3 *C*). Acetate, in fact, appeared to increase NKCC1 mRNA levels.

Butyrate selectively downregulates NKCC function in HT29 cells. We have demonstrated previously that parent HT29 cells display considerable levels of basal cotransport as assessed by bumetanide-inhibitable ^{86}Rb uptake. Despite the absence of a cAMP-regulated Cl^- efflux pathway in the HT29 plasma membrane, the cAMP agonist forskolin elicits a twofold increase in cotransporter activity (31). In these experiments, we found that treatment with sodium butyrate decreased peak cAMP-stimulated cotransporter activity with time (Fig. 4 *A*) and dose (Fig. 4 *B*) dependence that paralleled the changes seen at the mRNA level. Basal (unstimulated) cotransport was also reduced by butyrate, with forskolin able to elicit an approximately twofold increase in cotransport under each set of conditions. For example, 24-h treatment with 10 mM butyrate reduced basal cotransport from 10.3±1.7 to 6.22±0.7 nmol K^+/mg protein/min ($P < 0.05$) and forskolin stimulated cotransport from 25.1±2.2 to 15.0±1.2 ($P < 0.0004$, $n = 9$ and 12 for control and butyrate, respectively, with each experiment performed on triplicate monolayers). The effect

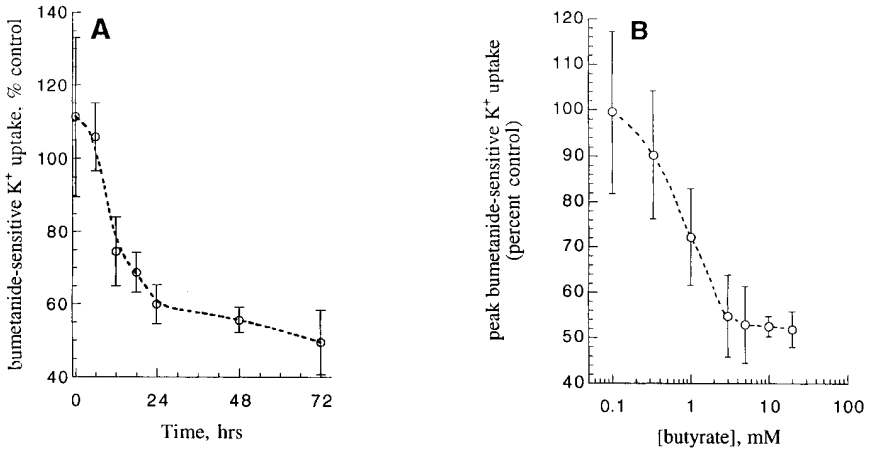

Figure 4. Butyrate decreases Na-K-2Cl cotransport in HT29 human intestinal epithelial cells. (*A*) HT29 monolayers were incubated in media in the absence or presence of 10 mM sodium butyrate for the time indicated on the *x* axis. Monolayers were then transferred to buffer solution without butyrate, as described in Methods. After stimulation with the cAMP agonist forskolin (10 μM), bumetanide-sensitive K^+ uptake was measured. Each data point represents the mean±SEM for 3–12 experiments performed on triplicate monolayers and is expressed as percent control. Control monolayers showed no time-dependent change over the 72-h experimental period (mean for all control monolayers = 24.9±1.4 nmol K^+/mg protein/min [n = 39 experiments performed on triplicate monolayers]). The data demonstrate that most of the downregulation response occurs between 6 and 18 h, with nearly maximal effects of butyrate achieved within ~24 h. The difference between control and butyrate-treated groups is significant ($P < 0.05$) by unpaired *t* test beginning at 18 h and confirmed by ANOVA with post-hoc Bonferroni/Dunn correction. (*B*) HT29 monolayers were incubated in media in the presence or absence of the indicated concentration of sodium butyrate for 24 h. As described in Methods, forskolin-stimulated bumetanide-sensitive K^+ uptake was measured. Data are expressed as mean percent control±SEM for triplicate monolayers, with similar results obtained in a second experiment. Nearly maximal effects on cotransport are seen at ~3 mM butyrate.

on the cotransporter appeared to be specific for this element of the secretory apparatus. The bumetanide-insensitive component of total ^{86}Rb uptake, which we showed previously to be largely ouabain-inhibitable and, thus, reflective of the activity of the Na-K ATPase (31), was not significantly inhibited by 72-h treatment with butyrate (e.g., K^+ uptake in the presence of bumetanide but absence of ouabain was 6.02±1.05 vs. 6.11±0.73 for forskolin-stimulated control and butyrate-treated cells, respectively [n = 4, NS]). Butyrate did not alter basal or cAMP-stimulated ^{86}Rb efflux (data not shown), measurements that largely represents conductive K^+ efflux pathways (32). Also, butyrate treatment did not induce a functional cAMP-regulated Cl^- efflux pathway; neither parent HT29 cells nor butyrate-differentiated cells displayed a significant increase in ^{125}I efflux in response to cAMP. The rate constant of ^{125}I efflux was 0.035±0.008 vs. 0.033±0.006 and 0.023±0.004 vs. 0.023±0.002 min^{-1} for unstimulated vs. forskolin in control and 72-h 10 mM butyrate-treated HT29 cells, respectively (each n = 4, NS).

Butyrate downregulates NKCC function and cAMP-regulated Cl^- secretory capacity in the T84 model of electrogenic Cl^- secretion. Although the HT29 cell model has proved useful to study enterocyte differentiation, its unpolarized nature limits its applicability to vectorial electrolyte transport (33). Therefore, we examined the effect of sodium butyrate on T84 cells, a human intestinal crypt-like epithelial line that is particularly well characterized as a model of electrogenic Cl^- secretion (22, 38). Treatment of T84 cells with 10 mM butyrate induced a time- and dose-dependent decrease in peak forskolin-stimulated I_{sc} (Fig. 5, *A* and *C*). The decrease in I_{sc} was specific for butyrate, and the SCFA acetate and proprionate in equimolar concentrations exerted no effect (Fig. 5 *B*). Theoretically, this decrease in secretory capacity could be due to inhibition of the cAMP-dependent activity of any or all of the four major components of Cl^- secretion. However, we found that 24-h sodium butyrate treatment did not significantly affect basal or cAMP-stimulated ^{125}I or ^{86}Rb efflux across either the apical or basolateral membranes (Fig. 6, *A* and *B*), respectively, suggesting that neither CFTR nor cAMP-dependent K^+ channels are impaired by prior butyrate treatment. However, butyrate did decrease basal and cAMP-stimulated bumetanide-sensitive ^{86}Rb uptake, consistent with results in the HT29 model (Fig. 6 *C*). Butyrate also decreased mRNA expression of NKCC1 (data not shown), as seen in HT29 cells. Butyrate did not decrease the bumetanide-insensitive component of ^{86}Rb uptake, which largely reflects activity of the ouabain-inhibitable Na-K ATPase (e.g., bumetanide-insensitive K^+ uptake was 7.75±0.34 vs. 6.70±0.46 and 11.85±1.68 vs. 7.85±0.98 nmol K^+/mg protein/min under unstimulated and forskolin stimulated conditions, respectively, for control monolayers vs. monolayers treated with 10 mM butyrate for 24 h [n = 4 experiments on triplicate monolayers, NS]).

Discussion

Stem cells at the base of the intestinal crypts yield the four main epithelial phenotypes: absorptive enterocytes (which constitute ~ 90% of the epithelial surface), goblet cells, enteroendocrine cells, and Paneth cells. During the course of their 3–5-d life span, enterocytes emerge from the proliferative crypt compartment and gradually acquire the specialized functions of digestion and absorption as they reach the nonproliferative villus compartment. Several reports have addressed the molecular mechanisms of upregulation of brush-border enzymes and transporters during enterocyte differentiation along the crypt-villus axis of the small intestine. For the most part, these studies have determined that the transition to the terminally differentiated villus enterocyte occurs via transcriptional activation of the genes encoding these apical membrane markers (8, 10, 12).

Comparatively little is known about the mechanisms involved in the disappearance of Cl^- secretory function during the course of enterocyte migration toward the villus tips. Previous studies have focused primarily on the patterns of expression of the CFTR gene, but there have been discrepancies between results obtained in vivo and in vitro. In situ hybridiza-

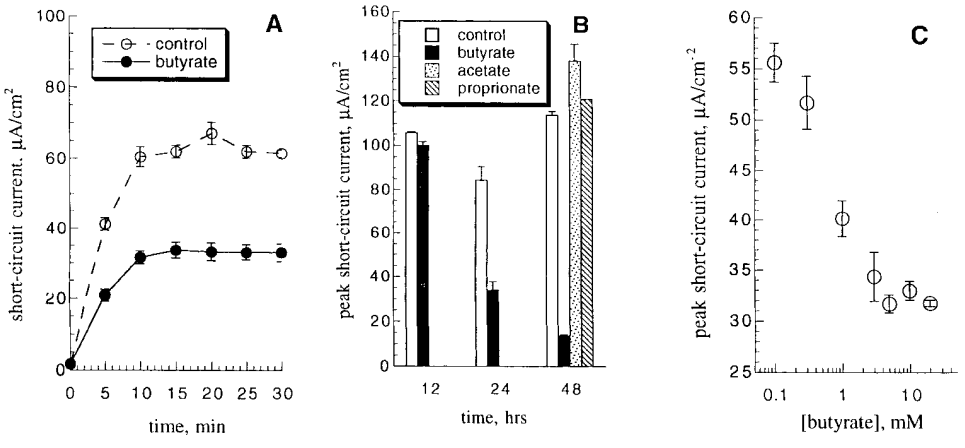

Figure 5. Butyrate inhibits cAMP-dependent Cl^- secretion in T84 human intestinal epithelial monolayers. (*A*) T84 monolayers grown on permeable supports were incubated in media for 24 h in the presence (*closed circles*) or absence (*open circles*) of 5 mM sodium butyrate. I_{sc} (which reflects net electrogenic Cl^- secretion) in response to 10 μM forskolin added at time zero was then determined as described in Methods. Data are mean±SEM for one representative experiment on triplicate monolayers ($P < 0.001$ by ANOVA) with similar results in three additional experiments. (*B*) Peak forskolin-stimulated I_{sc} responses were determined in control T84 monolayers or monolayers treated with either 5 mM sodium butyrate for 12, 24, or 48 h or with 5 mM sodium acetate or sodium proprionate for 48 h. Data represent mean±SEM for triplicate monolayers in each group. The differences between control and butyrate-treated monolayers is significant at $P < 0.05$ by unpaired t test at 24- and 48-h time points. Similar results were obtained in two additional experiments. (*C*) Monolayers were treated for 24 h with the indicated concentration of butyrate and peak forskolin-stimulated I_{sc} responses were determined. Data are mean±SEM for triplicate monolayers in each group.

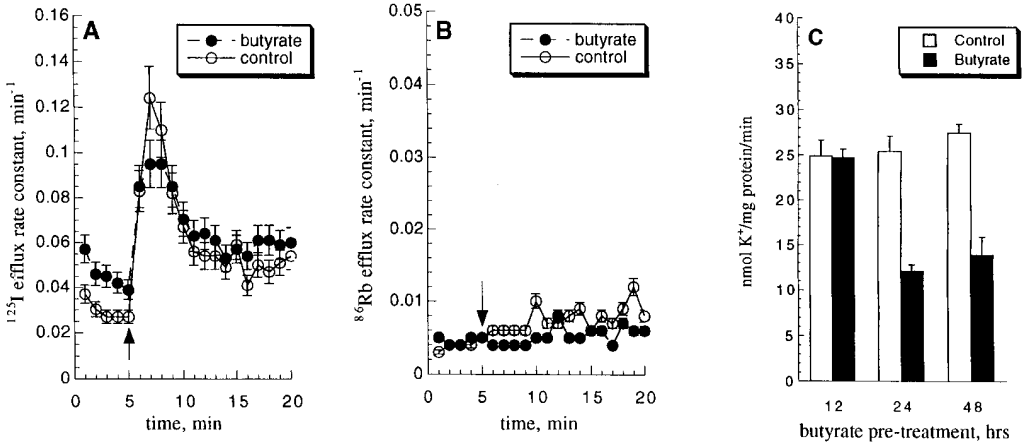

Figure 6. Butyrate inhibits cAMP-dependent Na-K-2Cl cotransport but not Cl^- and K^+ efflux pathways in T84 monolayers. Control T84 monolayers (*closed circles*) or monolayers treated with 10 mM sodium butyrate (*open circles*) for 24 h were preloaded with ^{125}I (*A*) and ^{86}Rb (*B*) as described in Methods. 1-min rate constants of ^{125}I efflux into the apical buffer and ^{86}Rb efflux into the basolateral buffer were then determined. After 5 min, 10 μM forskolin was added. Data are mean±SEM for three experiments performed on triplicate monolayers. The differences between forskolin-stimulated control and butyrate-treated monolayers are not statistically significant by unpaired *t* test and ANOVA with post-hoc testing for either isotope. (*C*) Control T84 monolayers or monolayers treated with 5 mM sodium butyrate for the indicated time periods were transferred to standard buffer solutions. After 20 min of stimulation with 10 μM forskolin, basolateral bumetanide-sensitive K^+ uptake was determined. Data are mean±SEM for four experiments performed on triplicate monolayers. By unpaired *t* test, differences at 24 and 48 h are significant ($P < 0.001$).

tion studies show the highest level of expression of CFTR in the crypts with a decreasing gradient of expression along the villus axis, suggesting that regulation in vivo is either transcriptional or due to changes in mRNA stability (11). In the duodenum and jejunum, a small population of cells highly positive for CFTR mRNA is scattered along the villus surface, but the function of these cells is unknown (11). In HT29 and Caco-2 models of differentiation an increase in CFTR gene expression is evident, primarily due to alterations in rates of mRNA degradation rather than transcription (14, 16). However, such regulation appears to be dissociated from changes in both CFTR protein levels and CFTR function (as assessed by cAMP-elicitable Cl^- efflux), probably due to complex alterations in the posttranslational processing of CFTR that occur with differentiation in vitro. CFTR expression and function does not appear to change as cells grow to confluence in the T84 line, which does not show evidence of spontaneous differentiation in culture (39).

The regulation of expression of basolateral components of Cl^- secretion during enterocyte differentiation has not been examined extensively. This study indicates for the first time that NKCC1 mRNA is expressed primarily in mammalian small intestinal crypt cells in vivo and is downregulated in an in vitro differentiation model. In situ hybridization studies will be useful to define more precisely cell type–specific patterns of NKCC1 gene expression, particularly within the crypt compartment. A recent abstract using immunofluorescence microscopy suggests a crypt-restricted pattern of expression of NKCC1 both in small and large rat intestine (40). It is unknown whether the scant population of CFTR-expressing villus enterocytes alluded to above also expresses high levels of NKCC1, although such data might yield clues as to the function of such cells. Giannella et al., using a similar cell separation technique in rat jejunum and ileum, reported an increasing gradient of mRNA expression for the Na-K ATPase along the crypt-villus axis (41). This pattern is also recapitulated by spontaneously differentiating Caco-2 cells (41). In our experiments, butyrate did not increase the functional activity of the Na-K ATPase, as assessed by bumetanide-insensitive K^+ uptake in both HT29 and T84 cells.

Augeron and Laboisse demonstrated that long-term butyrate treatment of HT29 cells induces biochemical and morphological changes of multiple differentiated intestinal epithelial cell types (42). More recently, short-term treatment with butyrate (days rather than weeks) has also been shown to induce both growth inhibition and differentiation in HT29 cells (13, 37). Previous studies have established that butyrate decreases [^{3}H]thymidine and cell counts while inducing IAP and villin expression, an effect that mirrors the transition from rapidly proliferating crypt cells to slowly proliferating, mature villus enterocytes (43). In this sense, butyrate-treated HT29 cells serve as a useful model of crypt-villus differentiation. This study solidifies this concept by demonstrating downregulation of steady-state mRNA levels for a basolateral transport element in a manner that recapitulates our findings with NKCC1 expression in crypt and villus cell populations from rat jejunum. Although T84 cells do not appear to differentiate spontaneously in culture, they do demonstrate some evidence of differentiation in response to butyrate. Similar to HT29 cells, T84 cells have been shown to respond to butyrate treatment with growth suppression and an increased expression of the differentiation marker alkaline phosphatase (44), and we show here that butyrate downregulates NKCC1 gene expression in both the HT29 and T84 cell lines.

NKCC1 downregulation in HT29 cells is not due to the decrease in cell growth induced by butyrate, since serum-starved or postconfluent cells showed no diminution in NKCC1 mRNA. In contrast to previous findings with IAP and villin, the effects of butyrate on NKCC1 mRNA are not abrogated by either cycloheximide or anisomycin and are, thus, independent of new protein synthesis (13). This early gene response to butyrate was also seen in regard to the induction of the cell cycle inhibitor, p21, in HT29 cells (Archer, S.Y., S. Meng, and R.A. Hodin, unpublished observations), and the vimentin and

metallothionein-1 genes in nonintestinal cells (45, 46). Butyrate does not apparently downregulate CFTR in HT29 cells; in fact, CFTR mRNA is induced by butyrate, although this increase occurs without a corresponding increase in cAMP-dependent Cl^- channel function. This finding illustrates the possibility of independent regulatory pathways for the apical and basolateral components of the Cl^- secretory apparatus.

Although apical membrane Cl^- channels, and in particular CFTR, are generally held to be the primary regulatory site for epithelial Cl^- secretion, it is clear that Cl^- uptake across the basolateral membrane must also increase to balance apical Cl^- exit and to preserve intracellular ion and volume homeostasis under changing physiologic demands. We have previously identified several conditions under which the regulation of the basolateral Na-K-2Cl cotransporter appears to limit the rate of transepithelial Cl^- transport (17, 18, 47). Our findings with butyrate-treated T84 cells further supports this concept. In this study, we found that butyrate did not decrease apical CFTR or cAMP-stimulated ^{125}I efflux, but it decreased both peak and sustained transepithelial secretory currents elicited by forskolin in a time- and dose-dependent manner. Thus, both HT29 and T84 lines respond to butyrate with a downregulation of NKCC1, and the loss of NKCC1 function during butyrate-simulated differentiation is associated with a diminution in Cl^- secretory capacity. This finding is also interesting in light of a recent report by Zhou et al., in which human CFTR was expressed in $CFTR^{-/-}$ mice under control of the fatty acid binding promoter (which is specific for villus rather than crypt enterocytes) (48). This manipulation restored cAMP-regulated Cl^- transport to a degree sufficient to prevent death from intestinal obstruction. However, secretory rates were not quantitatively restored compared to wild-type, a finding thought to be due to restricted basolateral Cl^- entry, and supported by the relative bumetanide insensitivity of forskolin-stimulated currents in the genetically rescued mice.

Butyrate is an SCFA by-product of bacterial fermentation of dietary fiber. Together with proprionate and acetate, these substances represent the major anionic species of colonic luminal fluid, with total SCFA concentrations typically $\sim$ 100 mM (butyrate is usually $\sim$ 20–30 mM) (49, 50). Butyrate is preferred over glucose as a metabolic substrate for colonic enterocytes. Its absence has been linked to the pathogenesis of several disease states including diversion colitis, and oral or rectal SCFA have been advocated as adjunct therapy in inflammatory bowel diseases, pouchitis, and diversion colitis (51). Dagher et al. reported recently that cAMP-dependent secretion in T84 monolayers was inhibited by $>$ 50% in the presence of 50 mM butyrate, an effect that was attributed to pH-independent acute inhibition of the apical membrane Cl^- conductance (52). In this study, we examined the effects of extended exposure to butyrate but carried out transport studies in the absence of this agent. Downregulation of NKCC1 gene expression and function were induced at substantially lower butyrate concentrations. The effects described by Dagher et al. are clearly complementary to ours and suggest that butyrate may well be an important negative regulator of colonic secretion both through direct short-term inhibition of apical Cl^- conductance and through long-term effects on basolateral Na-K-2Cl cotransport. This study also raises the intriguing possibility that butyrate may be useful therapeutically in certain diarrheal diseases by downregulating intestinal secretory capacity. Whether endogenous luminal butyrate plays an important role in enterocyte differentiation under physiologic conditions remains a matter of speculation. Nevertheless, this study suggests that modulation of the Cl^- secretory phenotype by altering gene expression of NKCC1 could represent a novel approach to the treatment of chronic diarrheal disorders.

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