Inhibition of epithelial chloride secretion by butyrate: role of reduced adenylyl cyclase expression and activity

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Resta-Lenert, Silvia, Francis Truong, Kim E. Barrett, and Lars Eckmann. Inhibition of epithelial chloride secretion by butyrate: role of reduced adenylyl cyclase expression and activity. Am J Physiol Cell Physiol 281: C1837–C1849, 2001.—Butyrate and other short-chain fatty acids (SCFAs) are found at high concentrations in the colonic lumen and affect multiple epithelial cell functions. To better understand how SCFAs regulate ion transport, we investigated the effects of SCFAs on Cl\(^-\) secretion in human colonic epithelial cell line T\(_{84}\). Butyrate inhibited Cl\(^-\) secretory responses to prostaglandin E\(_2\), forskolin, and cholera toxin. Other SCFAs were less effective or inactive. Reduced secretion was associated with decreased synthesis of the second messenger cAMP rather than increased degradation. Expression and activity of adenylyl cyclase were decreased by butyrate, whereas phosphodiesterase activity was unaffected and phosphodiesterase inhibition did not reverse the effects of butyrate on Cl\(^-\) secretion. Furthermore, butyrate decreased expression of the basolateral Na-K-2Cl cotransporter, indicating that it might modulate the secretory capacity of the cells. However, butyrate did not affect secretory responses to the calcium-dependent secretagogue carbachol, cAMP analogs, or uroguanylin, indicating that normal secretory responses to adequate levels of second messengers in butyrate-treated T\(_{84}\) cells are possible. These results show that butyrate affects several aspects of epithelial Cl\(^-\) secretion, including second messenger generation and expression of key ion transporters. However, these effects may not all be equally important in determining Cl\(^-\) secretion in response to physiologically relevant secretagogues.

In vitro, butyrate has been shown to induce cell differentiation in colonic and other epithelial cell lines (6, 10, 14, 17, 35), which is accompanied by alterations in gene expression via both translational and transcriptional mechanisms (35). In cultured colonic epithelial cells, butyrate increases the expression of alkaline phosphatase, mucins, the polymeric immunoglobulin receptor, and intercellular adhesion molecule-1 and modulates the expression and function of electrolyte transporters (16, 27, 30). Electroneutral absorption of NaCl is greatly stimulated by butyrate (5), whereas chloride secretion appears to be inhibited (12). The effect of butyrate on chloride transport was previously ascribed to downregulation of the Na-K-2Cl cotransporter NKCC1, whereas the expression and function of the apical cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel was not altered by the SCFA in vitro (30). Butyrate has also been shown to alter ion transporter function [e.g., Na/H exchanger (NHE2) by directly altering the pH microclimate in the apical region of enterocytes (19)].

In this study, we undertook a more detailed analysis of the effect of butyrate as a negative regulator of chloride secretion in the T\(_{84}\) cell line. We hypothesized that the effect of this SCFA on chloride secretion might be multifactorial and directed at other cellular events beyond downregulation of NKCC1. We demonstrate that butyrate has significant and irreversible inhibitory effects on chloride secretion evoked by prostaglandin (PG)E\(_2\), forskolin, and cholera toxin (CT). This can be ascribed predominantly to reduced intracellular cAMP production secondary to downregulation of the expression and activity of adenylyl cyclase (AC). NKCC1 expression is also reduced. In addition, we show that butyrate does not have any direct effect on phosphodiesterase (PDE) functions, because inhibition of PDEs does not reverse the effects of the SCFA on

SHORT-CHAIN FATTY ACIDS (SCFAs), including acetate, propionate, and butyrate, are by-products of bacterial fermentation of dietary fiber, i.e., undigested starches and proteins, in the colonic lumen. SCFAs are the predominant anions in the colon, with a total concentration exceeding 100 mM. Butyrate, the anion of the 4-carbon aliphatic carboxylic acid butyric acid, is present in the colonic lumen at concentrations of 10–30 mM. Colonocytes show a marked preference, above glucose, for butyrate as a source of energy, and 60–70% of the energy consumed by the colonic epithelium is derived from this SCFA (35). Impaired oxidation and/or decreased levels of butyrate may also play a role in the pathogenesis of colonic inflammation (7, 40). Local administration of butyrate has shown benefits in reducing colonic inflammation and alleviates diarrhea associated with inflammatory bowel disease, diversion colitis, and other intestinal inflammatory conditions (21, 36, 37).

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chloride secretion. Finally, the effects of butyrate on chloride secretion do not extend to calcium-dependent stimulation of this transport mechanism or to secretion evoked by the addition of cell-permeant cAMP analogs or the cGMP-dependent agonist uroguanylin.

MATERIALS AND METHODS

Cell culture. All studies were performed with T84 human colonic epithelial cells. Cells were grown in 50% DMEM, 50% F12 medium, supplemented with 5% heat-inactivated newborn calf serum, in an atmosphere of 95% air-5% CO2 at 37°C. To obtain polarized monolayers, cells not older than passage 22 were seeded onto 0.6-cm² Millicell HA filter inserts (Millipore, Bedford, MA) and grown until 2–3 days after confluence.

Electrophysiological studies. Vectorial ion transport was examined in modified Ussing chambers as described previously (4). The mucosal and serosal baths contained Ringer solution supplemented with glucose (115 mM NaCl, 25 mM NaHCO3, 0.4 mM KH2PO4, 1.2 mM MgCl2, 1.2 mM CaCl2, 5.6 mM glucose, adjusted to pH 7.4), which was gassed with 95% O2-5% CO2 at 37°C. The transepithelial voltage difference, and conductance were recorded for 10–15 min. After initial stabilization, baseline measurements of Ie, potential difference, and conductance were recorded for 10–15 min. Subsequently, agonists were added to the serosal and mucosal baths to stimulate chloride secretion and changes in electrophysiological parameters were recorded at regular intervals.

cAMP and cGMP assays. T84 cells, grown to confluence on Millicell HA filter inserts or in six-well tissue culture plates, were washed with warmed Hank’s balanced salt solution (HBSS) and incubated with PGE2, forskolin, or uroguanylin, as appropriate, at 37°C for various times. Cells were then placed on ice, washed with ice-cold HBSS containing 1 mM IBMX, and extracted with 500 μl/well ice-cold extraction buffer (67% ethanol, 33% HBSS, 1 mM IBMX) for 15 min. Cells were scraped from the well or filter, and the suspension was mixed and centrifuged at 12,000 rpm for 10 min at 4°C. Pellets and supernatants were dried separately under vacuum at room temperature. Dried pellets were resuspended in PBS, and cyclic nucleotide levels were assayed by enzyme immunoassay using commercially available kits (Cayman Chemical, Ann Arbor, MI; R&D Systems, Minneapolis, MN).

Immuno precipitation and immunoblot analysis. Cell monolayers were grown on 0.45-cm² Millicell filter inserts, and whole cell lysates were prepared as described previously (25). T84 monolayers were washed and incubated in cold lysis buffer [PBS, 0.1% Tween 20, 10 μM leupeptin, 10 μM PMSF, 10 μM phenylmethylsulfonyl fluoride (PMSF), 0.25 mM EDTA, 10 mM MgCl2, 10 mM EGTA, 0.5 mM EGTA, 10 μM leupeptin, 10 μM PMSF] on ice for 30 min. Cell membranes were isolated by centrifugation after lysis with hypotonic buffer (20 mM Tris base, 20 mM NaCl, 10 mM MgCl2, 1 mM vanadate, 1 μg/ml leupeptin, 100 μg/ml PMSF) as described previously (25). The filters were then scraped, and cell lysates were collected and centrifuged at 12,000 g for 10 min. Supernatants were removed, and protein concentrations were determined. The sample volumes were adjusted to provide equivalent protein concentrations. For immunoprecipitation, cell lysates were supplemented with additional PMSF (20 μM) and incubated overnight at 4°C with an optimal amount of specific antibody (anti-NKCC1 NH2-terminal polyclonal antibody, kindly provided by Dr. Christian Lytle, Univ. of California, Riverside, CA; anti-AC r-32 polyclonal antibody, Santa Cruz Biotechnology; or anti-CFTR (COOH-terminal epitope) monoclonal antibody, Genzyme, Cambridge, MA). The samples were then incubated with protein A agarose at 4°C for 1 h with constant mixing. The immunoprecipitates were collected by centrifugation at 15,000 g for 2 min, and the pellets were washed three times with lysis buffer. Samples were run on precast 7.5% or 4.5–15% acrylamide gels, and proteins were transferred onto a blotting membrane. After nonspecific binding sites were blocked with a PBS-Tween buffer containing nonfat dry milk, blots were incubated with optimal dilutions of primary (anti-NKCC1, anti-CFTR, or anti-AC) and secondary (horseradish peroxidase-labeled anti-mouse IgG or anti-rabbit IgG, as appropriate) antibodies for 30–60 min. Membranes were washed in PBS-Tween, incubated with chemiluminescent reagents (Amersham ECL System) for 2–3 min, and exposed to X-ray film for optimal periods.

Assays of AC and PDE activities. Cell membranes were prepared by nitrogen cavitation as using published protocols (24). Briefly, T84 cell monolayers were washed with HBSS, scraped into homogenization buffer (340 mM sucrose, 10 mM HEPES pH 7.4, 1 mM EDTA, 0.1 mM MgCl2, 2 mM NaN3, 1.25 mM PMSF, 10 mg/ml chymostatin), and centrifuged at 3,000 g for 5 min. The pellets were resuspended in 1 ml of homogenization buffer, and the suspensions were exposed to 200 psi of 100% nitrogen for 15 min at 4°C. After nitrogen cavitation, the homogenates were centrifuged at 3,000 g for 20 min to remove nuclei and cellular debris. The supernatants containing the membranes were collected and used to determine total protein content and enzymatic activities.

AC activity was determined as described by others (23) with the following modifications. Aliquots (20–50 μg total protein) of the membrane preparations were added to reaction buffer (50 mM Tris pH 7.5, 13 mM MgCl2, 2.6 mM EDTA, 25 mM phosphocreatine, 1 mg/ml creatine phosphokinase, 0.25 mM IBMX, 1 mM AMP, 1 × 106 cpm [32P]ATP, and 0.5 mM ATP) in a total volume of 50 μl. The membrane suspension was incubated for 15 min at 37°C with or without 100 μM forskolin. The reaction was terminated by adding 100 μl of stop solution (2% SDS, 40 mM ATP, 1.4 mM cAMP). Approximately 104 counts of [3H]cAMP were added to each reaction tube as a recovery marker. A double column separation to separate ATP from cAMP was performed according to Mittal (31). Each sample was added to a 1-ml bed of Dowex AG50W-X4 resin and eluted with 3 ml of water directly into scintillation vials to determine [α-32P]ATP. The columns were then placed over 0.6-g alumina columns and washed with 5 ml of water. Bound cAMP was eluted from the alumina columns with 4 ml of 100 mM imidazole directly into 20-ml scintillation vials filled with 10 ml of scintillation fluid. Radioactivity was determined in a scintillation counter.

PDE activity was determined as described by Fuhrmann et al. (15). Cell monolayers were washed twice with HBSS, collected into lysis buffer (PBS containing 10 mM HEPES, 1 mM MgCl2, 5 mM DTT, 10 μM trypsin inhibitor, 1 mM EGTA, 5 μM pepstatin, 10 μM leupeptin, 2 mM benzamidine), and sonicated on ice for 5 s. Cell lysates (20–50 μg protein) were added to the reaction buffer (60 mM Tris pH 7.4, 5 mM MgCl2, 1.25 mM CaCl2, 100 μM calmodulin, 105 cpm [3H]cAMP, and 0.5 μM cAMP) in a final volume of 200 μl. Approximately 104 cpm [α-32P]ATP were added as a recovery marker. Reactions were incubated for 15 min at 37°C. Samples were then cooled on ice for 10 min, 50 μl of 5'-nucleotidase (2 mg/ml Crotalis atrox venom in 400 mM
Tris, pH 8.5) were added, and the reaction mixtures were incubated at 37°C for 15 min. Samples were added to 1-ml beds of DEAE A-25 Sephadex columns (prewashed with 100 mM Tris buffer, pH 7.2) and eluted with 2 ml of distilled water directly into 20-ml scintillation vials. Radioactivity was determined in a scintillation counter.

Materials. Unless otherwise indicated, all chemicals were obtained from Sigma (St. Louis, MO). Purified cholera toxin (no. C3012) was obtained from Sigma and stored at 4°C before use.

Data analysis. Data are expressed as means ± SE. Differences between groups were analyzed by ANOVA or Student’s t-test, as appropriate. The statistical analysis comprised comparisons between butyrate and time-matched controls. P values of <0.05 were taken to indicate statistically significant differences.

RESULTS

Butyrate treatment inhibits PGE2-stimulated chloride secretion in T84 colonic epithelial cells. Monolayers of polarized T84 cells were treated with butyrate at various concentrations (1–50 mM) and for various times (1–72 h), washed, and mounted in Ussing chambers to assess baseline and stimulated chloride secretion. Changes in $I_{sc}$ in T84 cells are wholly representative of chloride secretion (3). T84 monolayers treated for up to 72 h with up to 50 mM butyrate showed no changes in transepithelial resistance (data not shown), indicating that these conditions had no effect on the viability or barrier integrity of the epithelial monolayers. Furthermore, baseline $I_{sc}$ was not different between butyrate-treated and control cells (data not shown), indicating that butyrate itself had no secretagogue activity.

We then tested whether butyrate treatment modulates chloride secretory responses by intestinal epithelial cells after stimulation with known agonists of this response, as suggested by previous studies (12, 30). Stimulation of control T84 monolayers with the potent secretagogue PGE2 strongly increased $I_{sc}$, and this response was inhibited in butyrate-treated cells in a concentration-dependent manner (Fig. 1A). A significant decrease in PGE2-stimulated $I_{sc}$ was observed as early as 3 h after butyrate addition with all doses tested, and maximal inhibition occurred after 48–72 h of butyrate treatment (Fig. 1B). Significant inhibition of PGE2-stimulated $I_{sc}$ was seen after addition of as little as 1 mM butyrate for 48 h, and maximal inhibition occurred at 50 mM (Fig. 1). The presence of butyrate for only 1 h at the beginning of the culture period, followed by further incubation without butyrate, had no significant effect on chloride secretion at 3 h at low concentrations of butyrate (1 and 5 mM), but even these concentrations were sufficient to significantly inhibit the PGE2-induced secretory response at 24–48 h after butyrate treatment using this washout design (Fig. 2). Thus, although continuous incubation with butyrate exerted a stronger inhibitory effect than transient exposure (cf. Fig. 1B), butyrate appeared to initiate its effects quite rapidly and irreversibly, although the functional consequences required a longer period to become fully apparent. To determine whether the effects of butyrate required its metabolism, we examined whether the poorly metabolized derivative isobutyrate was also able to inhibit chloride secretion. This fatty acid had no effect at 1–10 mM concentrations and only a modest effect at the 50 mM dose at some of the time points examined (Fig. 3). In contrast, propionate and acetate inhibited PGE2-stimulated chloride secretion at low concentrations, although even these SCFAs had inhibitory effects that were much less pronounced than those evoked by butyrate (Fig. 3). A 1-h incubation of T84 cells with isobutyrate, acetate, and propionate, followed by incubation in medium alone, did not result in significant inhibition of chloride secretion (Fig. 2).
Butyrate treatment downregulates expression of NKCC1 in T84 cells. The inhibition of the PGE2-induced secretory response in butyrate-treated T84 cells is consistent with the previous finding that secretory responses to forskolin are attenuated in butyrate-treated T84 cells (30), because both PGE2 and forskolin exert their effects on chloride secretion via increases in cellular cAMP levels. Consistent with the previous studies, we also found inhibition of forskolin-stimulated chloride secretion in T84 cells treated with butyrate (Fig. 4) but not in those treated with isobutyrate (not shown). Previous studies had also shown that butyrate treatment decreased epithelial expression of one of the crucial ion transporters, NKCC1, required for regulated chloride secretion in intestinal epithelia, suggesting that this event was limiting for forskolin-induced chloride secretory responses (30). We also observed decreased levels of NKCC1 in T84 cells at 24 and 48 h of butyrate treatment, although no significant decrease in expression was seen at 6 h either in whole cell lysates or in a membrane fraction (Fig. 5). The latter finding suggests that decreased NKCC1 expression in butyrate-treated T84 cells cannot be the sole mechanism underlying the decrease in chloride secretory response to PGE2, as this was already significantly inhibited after 3 h of butyrate treatment (Fig. 1B). Exposure of T84 cells to isobutyrate, acetate, and propionate had no significant effect on NKCC1 expression (data not shown).

Butyrate inhibits effect of CT in T84 cells. Chloride secretory responses in epithelial cells are known to be mediated by calcium or cyclic nucleotides. cAMP production by AC in T84 cells can be activated by different mechanisms: by agonists that interact directly with AC (e.g., forskolin), by agonists binding to G protein-coupled receptors (PGE2), or by agonists that interact with stimulatory G proteins (CT) or by uncoupling of inhibitory G proteins (Gin or Goa). The common result is an increased level of cAMP production by AC. To examine possible effects of butyrate at various points in this signaling pathway, we investigated responses to CT. CT is an arginine-specific ADP-ribosyl transferase that irreversibly inhibits Gs protein GTPase activity, thereby promoting accumulation of cAMP with subsequent sustained activation of chloride secretion by intestinal epithelial cells (13, 29). CT (30 pM) was added to monolayers for 2 h; the cells were then mounted in Ussing chambers, and ISc response was monitored. Butyrate significantly inhibited the effect of CT (30 pM, 2 h, 37°C) on chloride secretion by T84 cells (Fig. 6). These data are therefore consistent with the hypothesis that butyrate inhibits cAMP-mediated chloride secretory responses by modulating cyclic nucleotide generation.

Butyrate treatment has no effect on chloride secretory responses to carbachol or uroguanylin. Because NKCC1 expression was substantially inhibited in T84 cells after 24–48 h of butyrate treatment and this ion transporter is required for vectorial chloride secretion in response to multiple agonists (3), we reasoned that secretory responses to physiological secretagogues acting via mechanisms independent of cAMP might also be inhibited in butyrate-treated T84 cells. However, this was not the case, because peak chloride secretion in response to the Ca2+-dependent agonist carbachol was not significantly different in SCFA-treated vs. control cells, even at the highest concentration of bu-
Butyrate treatment also failed to significantly inhibit responses to the cGMP-dependent secretagogue uroguanylin (Fig. 8). These data show that butyrate treatment does not cause a general reduction in epithelial secretory responses but appears to affect cAMP-mediated secretory responses selectively, such as those observed after PGE2, forskolin, or CT stimulation. They further suggest that decreased NKCC1 expression is at most only partially responsible for the attenuation of PGE2-induced chloride secretion in butyrate-treated T84 epithelia.

Butyrate treatment of T84 cells does not alter secretory responses to cell-permeant cAMP. The decreased secretory response to PGE2 in butyrate-treated T84 cells could be due to reduced second messenger generation (i.e., lower cAMP levels after PGE2, forskolin, or CT stimulation), reduced secretory responses in the face of normal second messenger generation (i.e., a reduced capacity of the chloride secretory mechanism to respond to normal cAMP levels), or both. To test whether reduced responsiveness to cAMP plays a role, we stimulated T84 cells with three cell-permeant analogs of cAMP, dibutyryl cAMP-AM, dibutyryl cAMP, and 8-bromo-cAMP (8-BrcAMP). This approach bypasses the cellular signaling events required for CT-, forskolin-, or PGE2-stimulated cAMP generation and selectively tests whether the signaling pathways and ion transport processes downstream of cAMP generation are affected in butyrate-treated cells. Chloride secretion induced by dibutyryl cAMP-AM in butyrate-treated T84 cell monolayers was not significantly different from that in untreated control cells, even if cells were treated with butyrate for up to 72 h (Fig. 9A). Similarly, chloride secretion stimulated by dibutyryl cAMP or 8-BrcAMP was not markedly affected by butyrate.
Butyrate treatment (Fig. 9, B and C). Interestingly, at a low concentration (0.1 mM) and early time points, dibutyryl cAMP-stimulated chloride secretion was slightly inhibited in the presence of butyrate, but this was a transient effect rather than the progressive inhibition seen with PGE2. Responses to 8-BrcAMP were not altered at any time (Fig. 9, B and C). These data indicate that butyrate-treated T84 cells can secrete chloride normally in the presence of adequate levels of intracellular cAMP, suggesting that butyrate treatment of T84 cells has relatively little effect on the signaling pathways and ion transport processes downstream of cAMP generation. Consistent with this conclusion, and as shown by others (30), expression of the apically located cAMP-dependent chloride channel CFTR was not altered in butyrate-treated compared with control cells, as assessed by immunoblot analysis (data not shown).

Butyrate treatment decreases agonist-stimulated cAMP levels in T84 cells. The data described above indicated that butyrate had little effect on responses to the second messenger cAMP, which raised the possibility that the generation of this second messenger in response to the binding of PGE2 to its receptor is altered in butyrate-treated T84 cells. To test this, we determined cAMP levels after PGE2 stimulation in butyrate-treated and control T84 cells. As shown in Fig. 10, butyrate treatment resulted in reduced cAMP levels in response to PGE2 stimulation, with this effect being concentration dependent. The inhibitory effect on cAMP levels was observed within 3 h of butyrate treatment, although PGE2-stimulated cAMP levels continued to decrease further over time (Fig. 10A). Significant inhibition of PGE2-stimulated cAMP pro-

Fig. 4. Butyrate inhibits forskolin-induced chloride secretion in T84 cells. T84 cells were treated for 48 h (A) or the indicated times (B) with butyrate at 1 (•), 5 (▲), 10 (●), or 50 (▼) mM or were left untreated as controls (○). Monolayers were mounted in Ussing chambers, and $I_{sc}$ was determined after addition of forskolin (3 μM) to both sides of the monolayers. A: time course of $I_{sc}$ responses induced by forskolin addition. B: $\Delta I_{sc}$ responses induced by forskolin stimulation after various times of butyrate treatment, expressed as % of the value obtained in forskolin-stimulated time-matched control cells not treated with butyrate. Data are means ± SE; n = 6. Significant inhibitory effects of butyrate: **P < 0.01, ***P < 0.001 (ANOVA). In the case of overlapping symbols, the same level of significance (or lack thereof) applies to all; where error bars are not shown they are obscured by the symbol.

Fig. 5. Effect of butyrate on Na-K-2Cl cotransporter NKCC1 expression in T84 cells. T84 cells were treated with 10 mM butyrate, or left untreated as controls (F). Monolayers were immunoprecipitated from cell membrane fractions (A) or cell lysates (B) and detected by immunoblot analysis. A representative blot is shown at the top of each panel. Results of densitometric analysis of 4 separate experiments (means ± SE; expressed in arbitrary units, a.u.) are shown at the bottom: filled bars, control cells; open bars, butyrate-treated cells. **Significant difference (P < 0.01, ANOVA) vs. controls not treated with butyrate.
duction was observed even at the lowest concentration of butyrate tested (1 mM). These results indicate that butyrate treatment of T84 cells caused a decrease in second messenger generation in response to PGE2 stimulation, and this inhibition occurred with a time course that parallels the inhibitory effects of butyrate on epithelial chloride responses (compare Fig. 10 with Fig. 1B). Figure 10B shows cAMP production after PGE2 stimulation in cells that had been treated for only 1 h with butyrate (10 mM) and then incubated in fresh medium for up to 48 h before PGE2 addition. This experimental design resulted in a significant reduction in cAMP, although with delayed kinetics compared with continuous incubation. These findings were comparable to the studies of chloride secretion reported above (Fig. 2). Similarly, forskolin stimulation of T84 cells, i.e., using a direct agonist of AC, produced results comparable to those obtained with PG stimulation, both in terms of chloride secretion (Fig. 5) and cAMP production (data not shown).

To test the specificity of the effect of butyrate on cyclic nucleotide generation, we stimulated T84 cells with uroguanylin (1 μM, 30 min). This agonist stimulated an increase in cGMP levels from 10 ± 1 to 48 ± 5 pmol/mg protein in control T84 cells (means ± SE; n = 3, P < 0.01). In T84 cells treated for 48 h with 10 mM butyrate, uroguanylin stimulation increased cGMP levels from 8 ± 1 to 42 ± 5 pmol/mg protein (means ± SE; n = 3, P < 0.01). These data show that butyrate treatment had no significant effect on cGMP levels after uroguanylin stimulation, suggesting that the butyrate effects on cAMP generation are specific and do not extend to other cyclic nucleotides.

Effects of butyrate on epithelial cAMP levels and chloride secretion are not reversed by PDE inhibition.

Reduced cAMP levels after PGE2 stimulation of butyrate-treated T84 monolayers could be the result of decreased cAMP generation, increased cAMP degradation, or both. To test whether increased degradation contributes to decreased cAMP levels, we inhibited cAMP degradation in butyrate-treated T84 cells with the broadly specific PDE inhibitor IBMX and evaluated the effects on cAMP levels and chloride secretion in response to PGE2 or forskolin. As shown in Fig. 11, addition of IBMX to butyrate-treated T84 cells had relatively little effect on the butyrate-induced inhibition of PGE2-stimulated cAMP levels when tested after 48 h of butyrate treatment. Consistent with this finding, IBMX did not reverse the inhibitory effect of butyrate on PGE2-stimulated chloride secretion after 48–72 h of butyrate treatment (Fig. 12). At earlier times of

Fig. 6. Butyrate inhibits cholera toxin-induced chloride secretion in T84 cells. T84 cells were treated for the indicated times with butyrate at 10 mM (●) or were left untreated as controls (○). For the last 2 h before mounting in Ussing chambers, the cells were treated with cholera toxin (30 pM). Maximal chloride secretory responses to cholera toxin were then determined by monitoring Isc for an additional ≤90 min. Data are means ± SE; n = 4. ***Significant inhibitory effects of butyrate vs. time-matched controls (P < 0.001, ANOVA).

Fig. 7. Butyrate and other SCFAs have no effect on calcium-mediated chloride secretion in T84 cells. A: T84 cells were treated for 48 h with butyrate at 1 (●), 5 (▲), 10 (●), or 50 (▼) mM or were left untreated as controls (○). Monolayers were mounted in Ussing chambers, and Isc was determined after addition of carbachol (100 μM) to the basolateral side of the monolayers. Data are means ± SE; n = 6. No significant differences were observed between SCFA-treated and control cells under any of the conditions tested, as assessed by ANOVA.
Butyrate treatment increased PGE2-stimulated chloride secretory responses in butyrate-treated cells, as might be predicted on the basis of its ability to sustain PGE2-stimulated cAMP generation. This effect occurred to a similar extent in control cells, suggesting that IBMX did not specifically reverse the butyrate-induced inhibition of chloride secretion. Furthermore, IBMX treatment of T84 monolayers also failed to reverse the inhibitory effect of butyrate on forskolin-induced chloride secretion (D1sc at 24 h in mM/cm²: 17.4 ± 1.8 vs. 57.3 ± 2.2, butyrate vs. control after IBMX treatment, respectively; n = 6, P < 0.001). Together these data suggest that increased cAMP degradation is not responsible for the decrease in cAMP levels and chloride secretion after PGE2 or forskolin stimulation of butyrate-treated T84 cells.

To confirm these findings, we determined PDE activity directly in cell lysates from control and butyrate-treated T84 cells. Control cells had an IBMX-inhibitable PDE activity of 11.7 ± 0.7 pmol cAMP·min⁻¹·mg protein⁻¹ (means ± SE; n = 4). In comparison, T84 cells treated with 10 mM butyrate for 48 h showed 83.5 ± 12.0% of the IBMX-inhibitable PDE activity of controls analyzed in parallel (means ± SE; n = 4, P = not significant by Student’s t-test). Determination of PDE activities in lysates from cells stimulated for 10 min with PGE2 before the lysates were prepared yielded similar results (data not shown). These data indicate that butyrate-treated T84 cells exhibited no increase in PDE activity, giving further support to the conclusion that the reduction in PGE2-stimulated cAMP levels in butyrate-treated cells is not caused by increased cellular cAMP degradation.

Butyrate treatment downregulates activity and expression of epithelial ACs. Because cAMP degradation was not affected by butyrate treatment of T84 cells, we next investigated the possibility that cAMP synthesis was inhibited under these conditions. Membranes were prepared from control T84 cells and cells treated with 10 mM butyrate for 48 h and were assayed for AC activity after forskolin stimulation. Membranes from control cells showed 92 ± 20 pmol cAMP·min⁻¹·mg protein⁻¹ of forskolin-inducible AC activity (means ± SE; n = 4). In T84 cells treated with 10 mM butyrate for 48 h, membrane-prepared AC activity was 83.5 ± 12.0% of controls analyzed in parallel (means ± SE; n = 4, P = not significant by Student’s t-test). Together these data suggest that increased cAMP degradation is not responsible for the decrease in cAMP levels and chloride secretion after PGE2 or forskolin stimulation of butyrate-treated T84 cells.

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Butyrate treatment downregulates activity and expression of epithelial ACs. Because cAMP degradation was not affected by butyrate treatment of T84 cells, we next investigated the possibility that cAMP synthesis was inhibited under these conditions. Membranes were prepared from control T84 cells and cells treated with 10 mM butyrate for 48 h and were assayed for AC activity after forskolin stimulation. Membranes from control cells showed 92 ± 20 pmol cAMP·min⁻¹·mg protein⁻¹ of forskolin-inducible AC activity (means ± SE; n = 4). In T84 cells treated with 10 mM butyrate for 48 h, membrane-prepared AC activity was 83.5 ± 12.0% of controls analyzed in parallel (means ± SE; n = 4, P = not significant by Student’s t-test). Together these data suggest that increased cAMP degradation is not responsible for the decrease in cAMP levels and chloride secretion after PGE2 or forskolin stimulation of butyrate-treated T84 cells.

To confirm these findings, we determined PDE activity directly in cell lysates from control and butyrate-treated T84 cells. Control cells had an IBMX-inhibitable PDE activity of 11.7 ± 0.7 pmol cAMP·min⁻¹·mg protein⁻¹ (means ± SE; n = 4). In comparison, T84 cells treated with 10 mM butyrate for 48 h showed 83.5 ± 12.0% of the IBMX-inhibitable PDE activity of controls analyzed in parallel (means ± SE; n = 4, P = not significant by Student’s t-test). Determination of PDE activities in lysates from cells stimulated for 10 min with PGE2 before the lysates were prepared yielded similar results (data not shown). These data indicate that butyrate-treated T84 cells exhibited no increase in PDE activity, giving further support to the conclusion that the reduction in PGE2-stimulated cAMP levels in butyrate-treated cells is not caused by increased cellular cAMP degradation.
In contrast, membranes from T 84 cells treated with 10 mM butyrate for 48 h showed only 66.3 ± 3.5% of the inducible AC activity of controls (means ± SE; n = 4, P < 0.01 by Student’s t-test). These results indicate that butyrate treatment of T 84 cells caused a significant decrease in AC activity, which can provide an explanation for the decrease in cAMP generation after PGE2 stimulation in these cells. Decreased AC activity could be related to changes in expression, posttranslational events, or both. To assess expression of ACs in T 84 cells, we determined protein levels of total ACs by immunoblot analysis using an antibody that recognizes all AC isoforms. This approach was chosen because the enzyme activity data were obtained by stimulating membranes with forskolin, a pharmacological activator of the majority of AC isoforms (22). Cells treated with butyrate showed a significant decrease in total AC expression relative to controls, both in total cell lysates (Fig. 13) and in isolated membranes (data not shown). A significant decrease in AC expression was first observed after 3 h of butyrate treatment, consistent with the timing of the effect of butyrate on chloride secretion. AC expression reached a minimum by 24–48 h of butyrate treatment, with AC levels in butyrate-treated cells being only 15–20% of the levels in control cells as determined by densitometric analysis of the immunoblots (Fig. 13). These data suggest that decreased AC expression is responsible for decreased AC activity in butyrate-treated T 84 cells. In keeping with this conclusion, isobutyrate, acetate, and propionate had no significant effect on AC expression (Fig. 14). Moreover, when cells were incubated with butyrate for only 1 h and incubation was continued in fresh medium, AC levels still declined, albeit to a lesser extent than seen with continuous butyrate exposure (Fig. 15). Under these conditions, a significant decrease in AC expression was only seen at 24 and 48 h (Fig. 15).

DISCUSSION

This study demonstrates that butyrate inhibits cAMP-mediated chloride secretory responses in T 84 cells in a concentration- and time-dependent manner. The inhibitory effect of butyrate was first observed within 3 h and was paralleled by an inhibition of cAMP accumulation after PGE2 or forskolin stimulation and decreased AC expression. These findings suggest that decreased AC activity early after butyrate treatment of T 84 cells contributed significantly to the inhibitory effect of butyrate on cAMP generation in these cells.

Fig. 10. Effect of butyrate on PGE2-stimulated cAMP levels. A: T 84 cells were treated for the indicated times with butyrate at 1 (■), 5 (▲), 10 (●), or 50 (▲) mM or were left untreated as controls (○). Cells were then stimulated for 15 min with 10 μM PGE2, and cell extracts were assayed for cAMP. B: experiments similar to those shown in A, with the exception that the cells were treated with butyrate (10 mM) for 1 h only and then incubated with fresh medium for the times indicated before PGE2 stimulation. Results are means ± SE; n = 4. Significant inhibitory effects of butyrate: *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA). Data are means ± SE; n = 6. Basal levels of cAMP were similar in control and butyrate-treated cells (200 ± 50 vs. 220 ± 50 pmol/mg protein; n = 4, P = not significant). In the case of overlapping symbols, the same level of significance applies to all.

Fig. 11. Inhibition of phosphodiesterase activity does not reverse the inhibitory effect of butyrate on PGE2-stimulated cAMP levels. T 84 cells treated with 10 mM butyrate for 48 h (●) or untreated controls (○) were incubated with 0.5 mM IBMX (□) or buffer alone (●) for 30 min, after which time cells were stimulated with PGE2 (10 μM) for the times indicated on the x-axis. Cell extracts were prepared, and cAMP levels were determined. Results are means ± SE; n = 4. ***Significant differences (P < 0.001, ANOVA) vs. respective time-matched controls not treated with butyrate.
T84 cells is responsible for decreased cAMP production after PGE2 or forskolin stimulation, leading to a decreased chloride secretory response. In addition to the initial inhibitory effects, butyrate caused a more sustained inhibition of cAMP-mediated chloride secretory responses, which required 24–48 h to become fully apparent.

Besides inhibition of AC activity and cAMP generation, the later inhibitory effects of butyrate were paralleled by decreased NKCC1 expression. The latter finding is consistent with previous observations in HT-29 colonic epithelial cells (30). These studies showed that butyrate inhibition of forskolin-activated chloride secretion was accompanied by reductions in mRNA and protein expression of the basolateral Na-K-2CI transporter NKCC1, which is responsible for mediating basolateral uptake of chloride into epithelial cells (30). Although it is not clear whether the levels of NKCC1 or cAMP are limiting for chloride secretion in intestinal epithelia, it is possible that the effects of butyrate on both NKCC1 protein expression and generation of the second messenger cAMP produce a synergistic effect in inhibiting chloride secretion. On the other hand, our data show that the inhibitory effect of butyrate on chloride secretion does not extend to calcium- or cGMP-dependent secretory mechanisms or to secretion evoked by cell-permeant cAMP analogs. This suggests that the epithelial chloride secretory capacity in response to adequate levels of second messengers is not affected by butyrate, indicating that NKCC1 levels may not be limiting for the chloride secretory response under these conditions. Data showing an acute inhibitory effect of butyrate on preexisting chloride secretion (12, 36) also support a nonlimiting role of NKCC1 in the mechanism of action of SCFAs on chloride secretion. Furthermore, other effects of butyrate in modulating cellular ion transport have recently been described by Musch and colleagues (33) and others (5, 19, 36), underscoring the effects of this SCFA on sodium-hydrogen exchange and sodium-chloride absorption. By altering sodium and chloride absorption, in addition to effects on chloride secretion, the net effect of butyrate in vivo would be potentiated. Moreover, this study supports the evidence for unaltered CFTR expression in the presence of butyrate in intestinal epithelial cells (30). These data differ from the findings of...
Loffing et al. (28), which showed increased expression of CFTR after 6-day exposure to 5 mM butyrate or phenylbutyrate in Calu-3 airway epithelial cells. Organ-specific features, as well as the use of a different model (nystatin-permeabilized epithelial airway cells) and experimental design (6-day exposure to SCFAs), may explain the lack of concurrence with our observations.

Although our data align with previous findings by Matthews et al. (30), they differ from some of the conclusions proposed by Dagher et al. (12). Differences in the model (rat colon) and experimental design (acute exposure to SCFAs for both rat colon and nystatin-permeabilized T84 cells) may account for the discordant results, especially in relation to the interaction of butyrate with basolateral transport mechanisms and second messenger production. Our findings with CT are also internally consistent with our data and agree with previous work (2, 36). Furthermore, our data on the lack of effect of butyrate on cGMP-mediated chloride secretion and cGMP production are in agreement with other studies (11, 38) showing that butyrate does not interfere with cGMP production in response to guanylin or uroguanylin or in response to E. coli heat-stable endotoxin (STa) in T84 cells, although those authors also showed that cGMP distribution between intracellular and extracellular compartments is altered by the SCFA. Differences between our work and that of Dagher et al. (12), as well as a recent study by Charney et al. (8), which reported an inhibition of STa-induced chloride secretion and cGMP production by butyrate, may be ascribed to the model and experimental design (acute SCFA exposure) used by Charney for chloride secretion and cGMP determination (rat colon). Moreover, in intact tissues, cGMP may be produced in other cell types in addition to the epithelium and thereby evoke secondary effects on chloride secretion via the release of other mediators. The complexity of cell-cell interactions that regulate intestinal ion transport underscores the utility of a reductionist model such as the T84 cell line.

Butyrate treatment of T84 cells decreased the total activity and protein expression of ACs, suggesting that the downregulation of AC protein expression after butyrate is important for the decrease in cAMP production. Furthermore, AC expression was reduced, albeit with delayed kinetics, even in cells that had only a brief exposure to butyrate. In cardiac myocytes, changes in the expression of AC, rather than in G protein-coupled receptors or G proteins, have a greater influence on agonist-induced cAMP levels (18). Consistent with our findings regarding AC expression in T84 cells, butyrate inhibited total AC activity by 40–50% in GH1 pituitary cells (2), indicating that the effects of butyrate on AC activity may be conserved among different cell types. The observation that butyrate de-

![Fig. 14. Isobutyrate, propionate, and acetate have no significant effect on AC expression in T84 cells. T84 cells were treated with 10 mM SCFAs (isobutyrate, acetate, or propionate) for the indicated times or left untreated as controls. Total ACs were immunoprecipitated from cell lysates and detected by immunoblot analysis. A purified AC (Purif AC) protein mixture was used as a positive control (pos contr). A representative blot is shown at top; results of densitometric analysis of 6 separate experiments (means ± SE) are shown at bottom.](#)

![Fig. 15. One-hour exposure to butyrate inhibits AC expression in T84 cells, but with delayed kinetics. T84 cells were treated with 10 mM butyrate for 1 h and then incubated with fresh medium or left untreated as controls. Total ACs were immunoprecipitated from cell lysates and detected by immunoblot analysis. A purified AC (Pur AC) protein mixture was used as a positive control. A representative blot is shown at top. Below this, another blot shows that AC levels do not change over time in untreated control cells. Results of densitometric analysis of 6 separate experiments (means ± SE) are shown at bottom. Filled bars, controls; open bars, butyrate-treated cells. **Significant differences vs. time-matched controls not treated with butyrate (P < 0.01, ANOVA).](#)
creases total AC protein levels in T84 cells suggests that the expression of one or several of the more abundant AC isoforms is affected by butyrate. Little is currently known about the expression of specific AC isoforms in intestinal epithelial cells, although AC isoform expression has been well characterized in other systems. For example, brain cells express all of the AC isoforms (AC1–9) (9, 20, 26), whereas AC expression in other organs is limited to specific isoforms (26, 39).

We observed no increase in total PDE activity after butyrate treatment of T84 cells, suggesting that the decrease in cAMP levels by butyrate is mostly regulated by pathways that affect cAMP synthesis rather than its degradation. Consistent with these findings, butyrate also failed to affect PDE activity in mastocytoma cell lines, in which butyrate also induces cell differentiation (32). However, butyrate treatment increased PDE activity in GH1 pituitary cells (2), indicating that butyrate may have varying effects on total PDE activity in different cell types. The effect of butyrate on total PDE activity in GH1 cells suggests that the SCFA affects expression and/or activity of one or several PDE isoforms in that system. In fact, in a recent study by Zaharia et al. (41), acute treatment of rat colon and human colonic biopsy samples with 50 mM butyrate or other SCFAs led to an inhibition of cAMP-PDE activity, whereas cAMP-PDE activity was stimulated. Although our observations show no increase of PDE activity induced by 1- to 48-h exposure to butyrate in T84 cells, we cannot exclude the possibility that butyrate affects the expression and/or activity of specific PDE isoforms in T84 cells without altering total PDE activity in these cells and that early effects on cAMP levels could be related in part to modulation of specific PDE isoforms. This mechanism could explain the small inhibitory effect of butyrate that we observed with one cell-permeant form of cAMP (Fig. 9B). In fact, chloride secretion stimulated by dibutyryl cAMP at a lower concentration of 0.1 mM was significantly inhibited by butyrate (10 mM), an effect that was not reversed by addition of the PDE inhibitor IBMX (data not shown). Nevertheless, chloride secretory responses evoked by maximally effective concentrations of dibutyryl cAMP (1 mM) or the PDE-resistant analog 8-BrcAMP, were not affected by butyrate in any major way.

Butyrate induces growth arrest and cell differentiation in cultured colon epithelial cell lines (10, 17), which has led to its wide use to evoke an in vitro model of epithelial cell differentiation. Intestinal epithelial cell differentiation, which occurs in vivo during the migration of enterocytes from the crypts to the surface in the colon or from the crypts to the villi in the small intestine, is characterized by distinct molecular and phenotypical changes. These include increased expression of membrane hydrolases, such as sucrases and alkaline phosphatases (17), and changes in second messenger signaling (e.g., cAMP) (1). Our findings show that decreased PGE2 signaling and reduced AC expression and activity are likely to be part of the differentiation program of intestinal epithelial cells. Conversely, these data suggest that the state of differentiation of intestinal epithelial cells is important in the regulation of cAMP signaling in these cells, which may be relevant pathophysiologically. For example, mucosal inflammation is associated with epithelial damage, leading to increased epithelial regeneration that is characterized by an increase in the number of undifferentiated crypt cells (34). Under these conditions, the ratio of undifferentiated to differentiated epithelial cells is increased, which, on the basis of our present observations, would be predicted to be accompanied by an increased overall epithelial responsiveness to cAMP-mediated chloride secretion. Because fluid secretion can be considered a protective host function, the increased secretory capacity of the regenerating intestinal epithelium may help to reduce exposure of the epithelium to microbial and noxious agents, thus promoting healing. On the other hand, these responses may contribute to the adverse outcome of secretory diarrhea.

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