Butyrate increases apical membrane CFTR but reduces chloride secretion in MDCK cells

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green fluorescent protein; cystic fibrosis; gene expression; cystic fibrosis transmembrane conductance regulator; adenosine 3',5'-cyclic monophosphate

THE CYSTIC FIBROSIS transmembrane conductance regulator (CFTR) is a cAMP-activated Cl− channel that is targeted to the apical plasma membrane in many epithelial cells, including those in the kidney (5, 8, 31). In the kidney, CFTR is expressed in all nephron segments (31) and is important for transepithelial Cl− transport (12, 33) and enlargement of renal cysts in polycystic kidney disease (11). Mutations in CFTR are responsible for the genetic disease cystic fibrosis (CF) (22). Nearly 70% of individuals with CF express ΔF508-CFTR, a mutation that prevents the trafficking of CFTR from the endoplasmic reticulum to the plasma membrane (4, 35). Accordingly, cAMP does not stimulate Cl− secretion in CF epithelial cells. Although CF does not cause major alterations in renal function, individuals with CF do exhibit renal dysfunction including decreased ability to excrete a salt load, decreased ability to maximally dilute and concentrate urine, increased proximal tubule sodium reabsorption, and altered drug excretion (reviewed in Ref. 31). Recently, it was suggested that butyrate and the butyrate analog 4-phenylbutyrate may be useful therapeutic agents for treatment of CF in individuals expressing ΔF508-CFTR (3, 26, 27). These agents partially restored cAMP-activated Cl− secretion in nasal, bronchial, and pancreatic epithelial cells expressing ΔF508-CFTR by stimulating ΔF508-CFTR gene expression and increasing the amount of ΔF508-CFTR protein in the plasma membrane (3, 26, 27). The effect of butyrate on Cl− secretion by renal epithelial cells has not been reported.

The short-chain fatty acid sodium butyrate has numerous and diverse effects on cellular physiology including modulation of protein kinase and phosphatase activities (6, 23, 24, 28), stimulation of microtubule and microfilament formation (1), and transcriptional activation of numerous gene products (15) including heat shock proteins (9) and alkaline phosphatase (2). Because little is known about the effects of butyrate on CFTR-mediated Cl− secretion in renal epithelial cells, we examined the effects of sodium butyrate on the function and expression of wild-type CFTR in MDCK cells, a model for the renal distal tubule and collecting duct that secrete Cl−. Since expression of endogenous CFTR in renal epithelia is either low and difficult to detect or below the limit of detection of currently available techniques (5, 31), we fused CFTR to the green fluorescent protein (GFP-CFTR) and stably transfected MDCK cells with the GFP-CFTR cDNA (20). GFP, a 27-kDa protein from the jellyfish Aequorea victoria, generates a striking green fluorescence, is resistant to photobleaching, and does not require any exogenous cofactors or substrates to fluoresce (32). Previously, we demonstrated that fusion of GFP to CFTR does not alter CFTR function or localization (20). We report that sodium butyrate reduced Cl− secretion across MDCK cells, yet increased apical membrane GFP-CFTR expression 25-fold and increased apical membrane Cl− currents 30-fold. Although butyrate also increased Na-K-ATPase protein expression twofold, butyrate reduced the activity of the Na-K-ATPase by 55%. Our findings suggest that butyrate inhibits cAMP-stimulated Cl− secretion across MDCK cells in part by reducing the activity of the Na-K-ATPase.

METHODS

Cell culture. MDCK type I cells stably transfected with GFP-CFTR and parental untransfected MDCK I cells, which express low levels of endogenous CFTR (19), were grown in culture on Transwell filter-bottom cups as described previously (20). Sodium n-butyrate (5 mM; Sigma, St. Louis, MO)
was added to apical and basolateral cell culture chambers in complete cell culture media for 15–18 h. Fresh solutions of butyrate were prepared immediately before use for each experiment. Similar results, which are not pooled with presented data, were found in MDCK C7 cells, a clonal line of MDCK type I cells (generous gift of Dr. Hans Oberleithner) (10), stably transfected with GFP-CFTR.

Measurement of short-circuit current. Short-circuit current ($I_{sc}$) was measured across monolayers of MDCK cells grown on Transwell filter-bottom cups in the presence of amiloride ($10^{-5}$ M) in the apical solution to inhibit electrogenic sodium transport as described (14, 20). Under these conditions, cAMP-stimulated $I_{sc}$ across monolayers of MDCK cells is referable to CFTR-mediated Cl$^{-}$ secretion (20, 30). Butyrate was not present in bathing solutions during measurements of $I_{sc}$. A CAMP-stimulating cocktail ($100 \mu M$ 8-chlorophenylthiоПCAMP (CPT-CAMP), $100 \mu M$ IBMX, and $20 \mu M$ forskolin) was applied to both apical and basolateral solutions to stimulate $I_{sc}$. CPT-CAMP was obtained from Boehringer-Mannheim (Indianapolis, IN). IBMX and forskolin were purchased from Sigma (St. Louis, MO).

To examine the effect of butyrate on the activity of the Na-K-ATPase, the apical membrane was permeabilized with nystatin ($200 \mu g/ml$). $I_{sc}$ measured under these conditions represents the activity of the Na-K-ATPase, as described previously (25). To examine the effect of butyrate on apical membrane Cl$^{-}$ currents, the basolateral membrane was permeabilized with nystatin ($200 \mu g/ml$). When the basolateral membrane is permeabilized with nystatin, $I_{sc}$ measured in the presence of a transepithelial Cl$^{-}$ ion gradient directed from the apical to the basolateral solution ($140 \text{mM}$ vs. 14 mM: Cl$^{-}$ replaced on an equimolar basis with gluconate; Ca$^{2+}$ was increased from 0.5 to 2 mM in the gluconate-containing solution to maintain Ca$^{2+}$-activity similar in both solutions) represents the Cl$^{-}$ current across the apical membrane, as described previously (7, 13).

Confocal microscopy. Fluorescent images were acquired using a Zeiss Axioskop microscope (Thornwood, NY) equipped with a laser-scanning confocal unit (model MRC-1024; Bio-Rad, Hercules, CA), a 15-mW krypton-argon laser, and a ×63 PlanApochromat/1.4 NA oil-immersion objective. GFP fluorescence was excited using the 488-nm laser line and collected using a standard FITC filter set ($530 \pm 30$ nm). Propidium iodide fluorescence was excited using the 568-nm laser line and collected using a standard Texas Red filter set ($605 \pm 32$ nm). Images from vehicle- and butyrate-treated monolayers were collected using the same values for laser power, photomultiplier gain, iris, and black level. Acquired images were imported into Adobe Photoshop v3.0 for image processing and printing.

Cell surface biotinylation. Biotinylation of apical cell surface glycoproteins was performed as described (20). Following biotinylation, monolayers were solubilized in lysis buffer ($50 \text{mM Tris}-\text{HCl}, \text{pH} 8.0, 150 \text{mM NaCl}, 1\% \text{Nonidet P-40}$ and containing the Complete Protease Inhibitor cocktail; Boehringer Mannheim), scraped from filters, and spun at 14,000 g for 4 min to pellet insoluble material. Aliquots of cell lysates were removed for SDS-PAGE analysis, and the remainder of the supernatants were brought to a volume of 900 $\mu l$ with lysis buffer and precipitated with 100 $\mu l$ of a 50% slurry of streptavidin-agarose beads (Pierce, Rockford, IL) overnight at 4°C with end-over-end rotation. Beads were pelleted by brief centrifugation for 30 s at 14,000 g and washed three times with lysis buffer. Biotinylated proteins were eluted by boiling for 5 min in 50 $\mu l$ of Laemmli sample buffer ($0.24 \text{M Tris}-\text{HCl}, \text{pH} 8.9, 16\% \text{glycerol}, 0.008\% \text{bromphenol blue}, 5.6\% \text{SDS},$ and 80 $\mu M$ dithiothreitol).

SDS-PAGE and Western blotting. Cell lysates and biotinylated proteins were separated on 4–15% Tris-HCl gradient gels (Bio-Rad) and transferred to PVDF Immobilon membranes (Millipore, Bedford, MA). Membranes were blocked overnight at 4°C in 5% nonfat dry milk in TBS/0.02% Tween-20 and incubated with CFTR COOH-terminal (1:1,000) monoclonal antibody (Genzyme, Cambridge, MA), Na-K-ATPase α-subunit (1:1,000) monoclonal antibody (Upstate Biotechnology, Lake Placid, NY), or Na-K-2Cl cotransporter (1:10,000) monoclonal antibody T4 (17) (generous gift of Dr. Bliss Forbush III; Yale University, New Haven, CT) followed by anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5,000–1:10,000; Amersham, Arlington Heights, IL). Blots were developed by enhanced chemiluminescence (Amersham) using Hyperfilm ECL (Amersham). Densitometric analysis of band intensities was performed with public domain NIH Image v1.57 software.

Statistical analyses. Differences between means were compared by either paired or unpaired two-tailed Student’s t-test as appropriate using Instat v2.01 statistical software (GraphPad, San Diego, CA). Data are expressed as the mean ± SE. $P < 0.05$ is considered significant.

RESULTS

Butyrate inhibits electrogenic Cl$^{-}$ secretion in MDCK cells. We examined the effect of butyrate on basal and CAMP-stimulated $I_{sc}$ in parental, untransfected MDCK cells and in MDCK cells stably expressing GFP-CFTR. In parental untransfected cells, a CAMP-stimulating cocktail rapidly increased $I_{sc}$, which reached a peak at 2 min and declined to an elevated state above basal $I_{sc}$ (Fig. 1A; Table 1). Treatment of monolayers with butyrate ($5 \text{mM for 15–18 h}$) reduced basal and CAMP-stimulated $I_{sc}$ (Fig. 1A; Table 1). Thus butyrate inhibited CAMP-stimulated Cl$^{-}$ secretion mediated by endogenous CFTR. In cells stably transfected with GFP-CFTR, butyrate ($5 \text{mM for 15–18 h}$) also reduced basal and CAMP-stimulated $I_{sc}$ (Fig. 1B; Table 1). In contrast, acute butyrate treatment ($5 \text{mM for 15 min}$) had no effect on CAMP-stimulated $I_{sc}$ in GFP-CFTR-stable transfectedants (CAMP peak, $16.6 ± 3.3 \mu A/cm^{2}$ for vehicle-treated vs. $12.2 ± 2.1 \mu A/cm^{2}$ for butyrate-treated monolayers, $P > 0.05$; CAMP steady state, $6.3 ± 0.9 \mu A/cm^{2}$ for vehicle-treated vs. $5.9 ± 0.6 \mu A/cm^{2}$ for butyrate-treated monolayers, $P > 0.05$; n = 7–8 monolayers per group).1 Thus chronic, but not acute, butyrate treatment inhibited CAMP-stimulated Cl$^{-}$ secretion mediated by CFTR.

Effect of butyrate on GFP-CFTR expression. To begin to elucidate the mechanism(s) whereby chronic butyrate treatment decreased CTR-mediated Cl$^{-}$ secretion by MDCK cells, we examined the effect of butyrate on the expression and localization of GFP-CFTR by confocal microscopy, cell surface biotinylation, and Western blot analyses. In polarized cells grown on perme-
able filter supports, GFP-CFTR fluorescence was not detectable under control conditions (Fig. 2A). However, following 15–18 h of butyrate treatment, bright GFP-CFTR fluorescence was visible and localized to the apical plasma membrane region (Fig. 2B). Increased GFP-CFTR fluorescence following butyrate treatment is attributable to activation of the cytomegalovirus (CMV) promoter driving GFP-CFTR expression (20). Butyrate is known to activate transcription from many viral promoters, including the CMV promoter (15, 29, 34).

Due to the limited resolution of the confocal microscope in the vertical dimension (which corresponds to 0.5 µm under optimal scanning conditions), we cannot conclude with certainty that butyrate increased the amount of GFP-CFTR in the apical plasma membrane. It is possible that the GFP-CFTR observed in the vicinity of the apical membrane in butyrate-treated cells is actually localized to subapical vesicles less than 0.5 µm from the apical membrane, rather than in the apical plasma membrane. Therefore, we performed apical cell membrane biotinylation experiments to examine whether butyrate decreased cAMP-stimulated \( I_{sc} \) by altering the amount of GFP-CFTR in the apical membrane. As shown in Fig. 3, butyrate increased

### Table 1. Effect of 5 mM butyrate for 15–18 h on \( I_{sc} \) in MDCK cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>n</th>
<th>Basal ( I_{sc}, \mu A/cm^2 )</th>
<th>cAMP peak ( I_{sc}, \mu A/cm^2 )</th>
<th>cAMP elevated state ( I_{sc}, \mu A/cm^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-CFTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without Butyrate</td>
<td>4</td>
<td>2.7 ± 0.4</td>
<td>23.7 ± 3.9*</td>
<td>8.0 ± 1.9*</td>
</tr>
<tr>
<td>With Butyrate</td>
<td>5</td>
<td>1.7 ± 0.2†</td>
<td>8.6 ± 1.7*†</td>
<td>6.8 ± 2.4*†</td>
</tr>
<tr>
<td>Parental</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without Butyrate</td>
<td>4</td>
<td>2.2 ± 0.3</td>
<td>11.0 ± 1.4*</td>
<td>5.3 ± 0.9*</td>
</tr>
<tr>
<td>With Butyrate</td>
<td>4</td>
<td>0.1 ± 0.1†</td>
<td>2.7 ± 0.8*†</td>
<td>1.2 ± 0.1*†</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \) = no. of experiments. \( I_{sc} \), short-circuit current. * \( P < 0.05 \) compared with basal in same row. † \( P < 0.05 \) compared with same cell line without butyrate. GFP-CFTR, green fluorescent protein-cystic fibrosis transmembrane conductance regulator.
butyrate altered the expression of the Na-K-ATPase or Na-K-2Cl cotransporter, we performed Western blot studies. As shown in Fig. 4, Western blot analyses of whole cell lysates demonstrated that butyrate increased Na-K-ATPase expression nearly twofold. However, butyrate had no effect on Na-K-2Cl cotransporter expression (Fig. 4). These results cannot account for the butyrate-induced inhibition of cAMP-stimulated Cl− secretion.

Effect of butyrate on Na-K-ATPase activity and Cl− currents. In the next series of experiments, we tested the hypothesis that sodium butyrate reduced Cl− secretion across MDCK cells by inhibiting the activity of the Na-K-ATPase and/or apical CFTR Cl− channels. To examine the effect of butyrate on the function of the Na-K-ATPase, we permeabilized the apical membrane with nystatin and measured $I_{sc}$. Butyrate reduced the $I_{sc}$ attributed to the Na-K-ATPase from 52.2 ± 4.3 µA/cm² (n = 4) in vehicle-treated cells to 23.6 ± 1.9 µA/cm² in butyrate-treated cells (n = 4, P < 0.001). To examine the effect of butyrate on apical membrane Cl− channels, we permeabilized the basolateral membrane with nystatin (200 µg/ml) and measured $I_{sc}$ in the presence of a transepithelial Cl− ion gradient directed from the apical to the basolateral solution. Butyrate dramatically increased Cl− currents across the apical membrane from 9.2 ± 5.2 µA/cm² (n = 3) in vehicle-treated cells to 274.6 ± 31.4 µA/cm² in butyrate-treated cells (n = 3, P < 0.005). Taken together, these results suggest that butyrate inhibits CFTR-mediated Cl− secretion in part by reducing the activity of the Na-K-ATPase.

Effect of butyrate on basolateral membrane transport protein expression. Butyrate may inhibit cAMP-stimulated $I_{sc}$ across MDCK cells by reducing the expression and/or function of other transport proteins involved in transepithelial Cl− secretion such as the basolateral membrane Na-K-ATPase or Na-K-2Cl cotransporter. In MDCK cells, the Na-K-ATPase maintains a low intracellular concentration of Na+, which is important for providing the driving force for Cl− entry across the basolateral membrane via the Na-K-2Cl cotransporter; Cl− then exits the cell across the apical membrane through CFTR Cl− channels (30). To determine whether
DISCUSSION

The major new finding of this report is that chronic butyrate treatment reduced cAMP-stimulated Cl\(^{-}\) secretion across polarized MDCK cells expressing endogenous CFTR and MDCK cells stably expressing GFP-CFTR Cl\(^{-}\) channels. Paradoxically, sodium butyrate increased apical membrane GFP-CFTR protein expression 25-fold and increased apical membrane Cl\(^{-}\) currents 30-fold. Although butyrate also increased Na-K-ATPase protein expression twofold, it reduced the activity of the Na-K-ATPase by 55%. Our findings suggest that butyrate inhibits cAMP-stimulated Cl\(^{-}\) secretion across MDCK cells in part by reducing the activity of the Na-K-ATPase. We have made similar observations in human airway serous epithelial cells (16).

Several investigators have examined the effect of butyrate on wild-type CFTR-mediated transepithelial Cl\(^{-}\) secretion in nonrenal epithelial cells. In colonic T84 cells expressing endogenous wild-type CFTR, acute butyrate treatment (20–30 min) reduced cAMP-stimulated Cl\(^{-}\) secretion by inhibiting an apical membrane CFTR-like Cl\(^{-}\) conductance (7). Prolonged butyrate treatment (24 h) also inhibited Cl\(^{-}\) secretion in T84 cells; however, the effect of butyrate was mediated by downregulation of Na-K-2Cl cotransporter expression and activity without affecting CFTR expression and function (18). The apparent discrepancy between these studies on T84 cells may be attributed to differences in the dose or duration of butyrate treatment. In contrast, in the present study in MDCK cells, acute butyrate treatment had no effect on cAMP-stimulated Cl\(^{-}\) secretion, whereas prolonged butyrate treatment reduced cAMP-stimulated Cl\(^{-}\) secretion in part by inhibiting the Na-K-ATPase. Thus prolonged butyrate treatment inhibits CFTR-mediated Cl\(^{-}\) secretion in MDCK and T84 cells.

Our data suggest that butyrate inhibits Cl\(^{-}\) secretion across MDCK cells in part by reducing the activity of the Na-K-ATPase. In MDCK cells, the Na-K-ATPase maintains a low intracellular concentration of Na\(^{+}\), which is important for providing the driving force for Cl\(^{-}\) entry into the cell across the basolateral membrane via the Na-K-2Cl cotransporter. Cl\(^{-}\) then exits the cell across the apical membrane through CFTR Cl\(^{-}\) channels (30). By reducing the activity of the Na-K-ATPase, we speculate that butyrate increases intracellular Na\(^{+}\) concentration and thereby reduces Cl\(^{-}\) entry into the cell across the basolateral membrane via the Na-K-2Cl cotransporter. The decrease in Cl\(^{-}\) entry would reduce intracellular Cl\(^{-}\) concentration and thereby inhibit Cl\(^{-}\) secretion across the apical membrane, despite an increase in the amount of CFTR in the apical membrane. The cellular mechanism(s) whereby butyrate inhibits Na-K-ATPase activity is unknown. Butyrate has numerous and diverse effects on cell physiology including modulation of protein kinase and phosphatase activities (6, 23, 24, 28), stimulation of microtubule and microfilament formation (1), and transcriptional activation of numerous gene products (15), including heat shock proteins (9) and alkaline phosphatase (2). Additional experiments, beyond the scope of this report, are required to elucidate the cellular mechanism(s) whereby butyrate inhibits Na-K-ATPase activity in MDCK cells.\(^2\)

The observations in this report raise the question: why does butyrate allow cAMP to stimulate Cl\(^{-}\) secretion in CF cells? In CF epithelial cells CAMP has no effect on Cl\(^{-}\) secretion, because of an absence of CFTR in the apical plasma membrane. However, butyrate allows CAMP to activate Cl\(^{-}\) secretion in CF nasal, bronchial, and pancreatic epithelial cells expressing ΔF508-CFTR protein in the plasma membrane (3, 26, 27). The data in the present study appear to be at odds with the butyrate studies on CF cells. If butyrate also reduced Na-K-ATPase activity in CF cells, then one would predict that CAMP would not stimulate Cl\(^{-}\) secretion in CF cells, even though butyrate enhanced the amount of ΔF508-CFTR in the apical membrane. We propose that the effects of butyrate may be cell-type dependent such that butyrate may not change the activity of Na-K-ATPase in nasal, bronchial, and pancreatic epithelial cells. Thus the butyrate-induced increase in ΔF508-CFTR expression in the apical membrane would be sufficient to allow CAMP to stimulate Cl\(^{-}\) secretion. It is also possible that butyrate may reduce Na-K-ATPase activity in CF cells; however, the increase in apical Cl\(^{-}\) conductance, subsequent to the addition of ΔF508-CFTR to the membrane, is sufficient to increase Cl\(^{-}\) secretion even though Na-K-ATPase activity may fall with butyrate treatment. Additional studies are required to examine these possibilities.

The observation that butyrate has a negative effect on cAMP-stimulated Cl\(^{-}\) secretion by MDCK cells has potential implications for the use of butyrate to examine CFTR function in vitro and for the clinical use of butyrate in vivo. Butyrate-induced upregulation of CFTR expression from viral-based promoters in transfected cells may, as this study suggests, actually reduce Cl\(^{-}\) secretion. Clinical use of butyrate, although useful for treatment of patients with CF, urea cycle disorders, sickle cell disease, thalassemia, and cancer (21), may actually compromise wild-type CFTR function in kidney epithelia.

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\(^2\) Although sodium butyrate had no effect on Na-K-2Cl protein expression on MDCK cells, it is possible that butyrate reduced the activity of the cotransporter. Such an effect would be expected to contribute to the fall in CAMP-stimulated Cl\(^{-}\) secretion observed in butyrate-treated cells. Additional studies are required to determine whether butyrate inhibits the activity of the Na-K-2Cl cotransporter.
REFERENCES


