Regulation of butyrate uptake in Caco-2 cells by phorbol 12-myristate 13-acetate

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Alrefai, W. A., S. Tyagi, R. Gill, S. Saksena, C. Hadijaipou, F. Mansour, K. Ramaswamy, and P. K. Dudeja. Regulation of butyrate uptake in Caco-2 cells by phorbol 12-myristate 13-acetate. Am J Physiol Gastrointest Liver Physiol 286: G197–G203, 2004.—Butyrate and the other short-chain fatty acids (SCFAs) are the most abundant anions in the colonic lumen. Also, butyrate is the preferred energy source for colonocytes and has been shown to regulate colonic electrolyte and fluid absorption. Previous studies from our group have demonstrated that the HCO₃⁻/SCFA⁻ anion exchange process is one of the major mechanisms of butyrate transport across the purified human colonic apical membrane vesicles and the apical membrane of human colonic adenocarcinoma cell line Caco-2 and have suggested that it is mainly mediated via monocarboxylate transporter-1 (MCT-1) isoform. However, little is known regarding the regulation of SCFA transport by various hormones and signal transduction pathways. Therefore, the present studies were undertaken to examine whether hydrocortisone and phorbol 12-myristate 13-acetate (PMA) are involved in a possible regulation of the butyrate/anion exchange process in Caco-2 cells. The butyrate/anion exchange process was assessed by measuring a pH-driven [¹⁴C]butyrate uptake in Caco-2 cells. Our results demonstrated that 24-h incubation with PMA (1 μM) significantly increased [¹⁴C]butyrate uptake compared with incubation with 4μM PMA (inactive form). In contrast, incubation with hydrocortisone had no significant effect on butyrate uptake in Caco-2 cells compared with vehicle (ethanol) alone. Induction of butyrate uptake by PMA appeared to be via an increase in the maximum velocity (Vₘₐₓ) of the transport process with no significant changes in the Kₘ of the transporter for butyrate. Parallel to the increase in the Vₘₐₓ of [¹⁴C]butyrate uptake, the MCT-1 protein level was also increased in response to PMA incubation. Our studies demonstrated that the butyrate/anion exchange was increased in response to PMA treatment along with the induction in the level of MCT-1 expression in Caco-2 cells.

short-chain fatty acid; monocarboxylate transporter 1; human intestine; protein kinase C

SHORT-CHAIN FATTY ACIDS (SCFAs) butyrate, acetate, and propionate are products of the bacterial fermentation of undigested proteins and carbohydrates by the colonic flora (44). These monocarboxylate weak acids comprise the majority of the anions and the osmoles (≈150 mM) in the colonic lumen and contribute to many colonic epithelial cell activities (32). SCFAs, particularly butyrate, are the preferred source of energy for the colonocytes (12, 39). Also, previous studies (17, 21, 31, 45) showed that butyrate induces apoptosis, reduces cell proliferation, and promotes cellular differentiation and hence functions as an antitumorigenic factor in the colon. Butyrate has also been implicated in suppressing mucosal inflammation (22, 27). In addition, SCFAs are known to regulate fluid and electrolyte absorption in the colon (32). Butyrate is known to stimulate colonic electroneutral NaCl absorption and to inhibit chloride secretion (4, 35). Furthermore, recent studies (25) demonstrated that butyrate stimulates promoter activity and expression of the apical Na⁺/H⁺ exchanger (NHE3) in human adenocarcinoma cell line Caco-2. These previous data clearly indicate the importance of SCFAs for the colonic epithelial integrity and function.

With respect to the mechanisms of SCFA absorption by the luminal membrane of the colonocytes, previous studies (8–10, 19, 20, 30, 42) have indicated that two major mechanisms appear to be involved in SCFA transport: 1) nonionic diffusion of protonated SCFAs (8, 10) and 2) carrier-mediated transport of SCFA ions (19, 20). In the first hypothesis, it was proposed that H⁺ available in the colonic lumen from the activity of the apical NHE3 would protonate SCFAs into nonionic forms that readily permeate the apical membrane of the colonocytes. However, with an acidic dissociation constant of 4.8, the majority of SCFAs are expected to be ionized at the physiologic pH in the colonic lumen. In this regard, a number of recent studies (19, 30, 37) have provided compelling evidence for the presence of carrier-mediated pathways for ionized SCFA absorption by the colonocytes. However, the relative contribution of each pathway in SCFA absorption in vivo is not entirely clear.

Previous studies from our laboratory utilizing purified apical membrane vesicles have demonstrated the presence of SCFA⁻/HCO₃⁻ exchange processes in the human ileum and colon (19, 20). Similar exchange activity has also been shown in apical membrane vesicles of pig and rat distal colon (30, 37). The described SCFA⁻/HCO₃⁻ exchange process was insensitive to inhibition by DIDS, ruling out the possible involvement of the luminal membrane Cl⁻/HCO₃⁻ exchanger in the carrier-mediated SCFA uptake process. However, analog competition experiments and the inhibitor profiles of the described carrier-mediated SCFA transport strongly suggested the involvement of monocarboxylate transporter-1 (MCT-1) in this process (7, 11, 38). Additionally, studies from our laboratory utilizing the antisense technique further confirmed the involvement of MCT-1 in butyrate uptake in Caco-2 cells (16). MCTs represent a gene family of at least nine isosforms expressed in a...
variety of tissues and mediate the electro-neutral transport of 1-lactate and pyruvate across the plasma membrane (18, 34). Studies have shown the expression of MCT-1 isoform in rat and pig small intestine and colon (28, 38). Pig MCT-1 cDNA was able to transport butyrate as well when expressed in Xenopus oocytes, and its protein product was localized to the apical membrane of the colonocytes (38). Additionally, studies from our laboratory and others demonstrated that human MCT-1 polypeptide was localized to apical membrane of the human colonic epithelial cells (14).

Previous studies (3, 23, 46, 47) have shown that various hormones and signal transduction pathways modulate a number of intestinal apical membrane ion transporters. Hydrocortisone was shown to upregulate the function and expression of intestinal NHE3. Also, a recent study from our laboratory (1) demonstrated that NHE3 expression and function were increased by long-term incubation with phorbol 12-myristate 13-acetate (PMA) and downregulation of PKCα in Caco-2 cells. However, very little is known about the regulation of the human intestinal luminal carrier-mediated butyrate absorption.

Therefore, the present studies were undertaken to investigate the possible regulation of butyrate uptake by hydrocortisone and chronic exposure to PMA utilizing Caco-2 cells as an in vitro experimental model. Our data demonstrated that 24-h exposure to PMA but not hydrocortisone increased butyrate uptake in Caco-2 cells. The upregulation of butyrate uptake appeared to be due to an increase in the maximum velocity ($V_{\text{max}}$) value with no alteration in the apparent affinity for butyrate. In parallel, PMA treatment also resulted in an increase in the level of MCT-1 protein expression in Caco-2 cells, suggesting the involvement of MCT-1 in the upregulation of butyrate uptake by PMA.

MATERIALS AND METHODS

Cell Culture

Caco-2 cells were obtained from American Type Culture Collection and cultured at 37°C in an atmosphere of 5% CO₂. Cells were maintained in DMEM with 4.5 g/l glucose, 2 mM l-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 μg/ml gentamicin, 10 mM HEPES, 1% essential and nonessential amino acids, and 20% FBS. For the uptake experiments, cells from passages 25 and 50 were plated in 24-well plates at a density of 2 × 10⁴ cell/cm². Confluent monolayers were then used for the transport experiments at day 10 postplating. To study the effect of hydrocortisone or PMA, cells were rendered quiescent by serum removal for 12 h before study, and the indicated amount of hydrocortisone and PMA was added for 24 h. Control cells were treated with ethanol or the inactive form of phorbol ester, 4øPMA.

[¹⁴C]Butyrate Uptake

Butyrate uptake experiments were performed as previously described by us (16). Briefly, cells were equilibrated at room temperature for 20 min at the time of the experiment and then washed and incubated for 30 min at 25°C with HBSS containing in mM: 1.3 CaCl₂, 5.4 KCl, 0.44 K₂HPO₄, 0.4 MgSO₄, 0.4 Na₂HPO₄, 4 NaHCO₃, 0.5 MgCl₂, 135 choline Cl, and 10 HEPES, pH 7.4. Cells were then washed and incubated with HBSS, pH 6.5, containing the indicated concentration of [¹⁴C]butyrate (Perkin Elmer, Boston, MA) for the designated period of time. To terminate the transport process, cells were washed twice with ice-cold PBS. Finally, the cells were solubilized with 0.5 N NaOH for 4 h. The protein concentration was measured by the method of Bradford (5), and radioactivity was counted by a liquid scintillation analyzer Tri-CARB 1600-TR (Packard Instruments, Downers Grove, IL). Because the 5-min time point was in the linear range of the uptake of [¹⁴C]butyrate, the uptake was measured at 5 min and was expressed as nanomoles per milligram protein per 5 min. For the kinetic experiments, the uptake values were analyzed for simple Michaelis-Menten kinetics utilizing a nonlinear regression data analysis from a computerized model (GraphPad; Prism, San Diego, CA).

Protein Extraction and Western Blot Analysis

Total protein extracts were prepared from Caco-2 cells treated as indicated above. Cells were washed twice with ice-cold PBS and then incubated for 30 min at 4°C with lysis buffer containing (in mM) 150 NaCl, 5 EDTA, 1 Na othrovanadate, 1 PMSF, and 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 20 Tris-HCl, pH 7.4, and protease inhibitor mixture (Complete; Roche Diagnostics, Mannheim, Germany). Cells were then centrifuged at 13,000 g at 4°C for 30 min, the pellet was discarded, and the protein concentration in the clear supernatant was determined by the method of Bradford (5). Fifty micrograms of total protein were boiled for 5 min in Laemmli buffer and subjected to 10% SDS-PAGE and then transferred to nitrocellulose membrane at 4°C. The blots were first blocked for 1 h with the blocking buffer of PBS buffer containing 5% nonfat dry milk and 0.2% IGEPAL (Sigma Aldrich, St. Louis, MO) and then transferred to nitrocellulose membrane at 4°C. The blots were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Amersham, Arlington Heights, IL) with a dilution of 1:2,000 in the blocking buffer containing 1% nonfat dry milk. Blots were then again washed extensively with the same solution. The bands were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham).

RESULTS

Effect of PMA and Hydrocortisone on Butyrate Uptake in Caco-2 Cells

Several hormones and protein kinases have been demonstrated to be involved in the regulation of function and expression of a number of electrolyte transporters in various tissues and cell types (1, 47). Therefore, we examined the possible role of hydrocortisone as well as PMA in the regulation of butyrate uptake in Caco-2 cells. As shown in Fig. 1A, 24 h incubation with 1 μM PMA significantly increased the uptake of butyrate in Caco-2 cells by 35 ± 1.8% compared with incubation with the inactive form 4øPMA. The uptake values in Caco-2 cells treated with 4øPMA were not significantly different from the uptake values in untreated cells (data not shown). In contrast, incubation with 1 μM hydrocortisone for 24 h had no significant effect on butyrate uptake in Caco-2 cells (Fig. 1B). Furthermore, Fig. 2 shows the effect of PMA on butyrate uptake exhibited a dose-dependent response because the significant increase in the uptake was only seen when the cells were incubated with 1 μM but not 1, 10, or 100 nM of PMA. These data indicate the involvement of PMA but not hydrocortisone in the regulation of butyrate uptake in Caco-2 cells.

Effect of PMA on Kinetics of Butyrate Uptake in Caco-2 Cells

To further examine the effect of phorbol ester on butyrate transport in Caco-2 cells, we investigated the effect of 24 h incubation with 1 μM PMA on the kinetic parameters of
butyrate uptake. As shown in Fig. 3, incubation with PMA significantly increased (32%) the V_max of the transport process compared with cells incubated with 4αPMA (inactive form) for 24 h. [14C]butyrate uptake (5 mM) was then assessed for 5 min as described in MATERIALS AND METHODS. Results are presented as %control and represent means ± SE of 8 determinations from 4 separate experiments. *P < 0.05 compared with control (4αPMA). Control value for cell incubated with 1 μM 4αPMA was 24.1 ± 2.2 nmol/mg protein 15 min 1.

Involvement of MCT-1 in the Regulation of Butyrate Uptake by PMA

Effect of lactate. Previous studies from our laboratory (16) have suggested that the monocarboxylate transporter MCT-1 represents one of the major routes for butyrate transport in the human colon and Caco-2 cells. Studies (33) also showed that MCT-1 has stereoselective substrate specificity with more affinity for L- compared with D-lactate. To further investigate the involvement of MCT-1 in the intestinal butyrate transport, we studied the effects of both L- and D-lactate on butyrate uptake in Caco-2 cells. As shown in Fig. 4, the presence of 10-fold excess of L- but not D-lactate significantly inhibited butyrate uptake in Caco-2 cells, providing further evidence for the involvement of MCT-1 in the uptake process. To investigate the role of MCT-1 in the regulation of butyrate uptake by PMA, we examined the effect of PMA on the lactate-sensitive butyrate uptake in Caco-2 cells. Figure 5 demonstrates that lactate-sensitive butyrate uptake was significantly increased in Caco-2 cells by 55% in response to 24-h exposure to PMA.
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PMA compared with exposure to 4αPMA. These data suggest that MCT-1 is responsible for the PMA-mediated increase in butyrate uptake in Caco-2 cells.

Effect of PMA on the expression of MCT-1 in Caco-2 cells. Because 24-h exposure to PMA increased the $V_{\text{max}}$ of butyrate uptake in Caco-2 cells, we examined the effect of PMA incubation on the protein level of MCT-1. Figure 6 shows a representative Western blot showing the expected size bands of both human MCT-1 (~45 kDa) and β-actin utilizing specific antibodies and protein lysates from Caco-2 cells treated with 1 μM of PMA or 4αPMA. Figure 6 shows that, parallel to an increase in the $V_{\text{max}}$ value for butyrate uptake, the level of MCT-1 protein was also significantly increased (~5-fold) in Caco-2 cells treated for 24 h with PMA compared with cells incubated with 4αPMA. These results further support the conclusion that MCT-1 is responsible for the increase in butyrate uptake in response to PMA incubation.

Substrate-Induced Butyrate Uptake in Caco-2 Cells

It has been previously shown that the expression and activity of PKC were decreased in response to chronic incubation of Caco-2 cells with butyrate (36). Also, substrate-induced function and expression of MCT-1 has previously been shown in the AA/C1 colonic epithelial cell line (11). Therefore, similar to the above study, in the present study we also examined whether chronic incubation with butyrate will stimulate [14C]butyrate uptake in Caco-2 cells. As depicted in Fig. 7, 24-h incubation with 5 mM butyrate significantly stimulated butyrate uptake to an extent similar to that observed by PMA, indicating that prior observations of substrate-induced changes in butyrate activity as reported by Cuff et al. (11) could have been secondary to PKC downregulation by butyrate as previously shown (36).

Effect of Acute PMA Treatment on Butyrate Uptake

Short-term exposure of PMA is well known to activate PKC in Caco-2 cells (24). However, previous studies (24) demonstrated that long-term exposure to 1 μM PMA lead to a downregulation of PKC expression and function. In this regard,
activation of PKC in response to short-term exposure to PMA (15 and 30 min) failed to have any significant effect on butyrate uptake in Caco-2 cells (18.14 ± 0.56 for 15 min with 4nM PMA vs. 17.86 ± 0.84 nmol·mg protein−1·min−1 for 15 min with PMA; 18.63 ± 0.7 for 30 min with 4nM PMA vs. 18.0 ± 1.5 nmol·mg protein−1·min−1 for 30 min with PMA). These results, therefore, suggest that the stimulation of butyrate uptake in response to PMA treatment in Caco-2 cells is secondary to downregulation rather than by the stimulation of PKC activity.

**DISCUSSION**

Previous studies from our laboratory (16) have demonstrated that the Caco-2 cell line can function as a suitable model to study butyrate uptake and suggested that MCT-1 played a major role in the butyrate uptake process in these cells. The present studies were designed to investigate the possible regulation of butyrate uptake by hydrocortisone and PMA and to further examine the role of MCT-1 in the regulation of butyrate transport in Caco-2 cells. Our data show that 24-h exposure to PMA but not hydrocortisone significantly stimulated the uptake of [14C]butyrate in Caco-2 cells. Stimulation of the uptake by PMA was dose dependent and was associated with an increase in the Vmax of the uptake with no alteration in the apparent Km for butyrate. Stimulated uptake was inhibited with 10-fold excess of cis-concentration of l- but not d-lactate. In parallel to the increase in the Vmax of butyrate uptake, our results show that the level of MCT-1 protein was also significantly increased (~5-fold) after 24-h treatment with PMA, indicating the involvement of MCT-1 in this regulation.

SCFAs make up the majority of anions in the colonic lumen and are the preferred source of energy for the colonocytes (32). SCFAs play an important role in the integrity and function of the colonic epithelium (12, 17, 21, 31, 32, 39, 45). Therefore, it is of great importance to explore the regulation of their uptake by the colonocytes. In this regard, glucocorticoid hormone has been shown to have major effects on several electrolyte transport activities. Previous in vitro and in vivo studies (3, 23, 46, 47) have shown that glucocorticoid increased Na+/H+ exchange activity (NHE) and NHE3 isoform expression in various species and cell types. However, present studies indicate that hydrocortisone treatment had no significant effect on butyrate uptake in Caco-2 cells.

In this regard, phorbol esters, analogs of diacylglycerol, acutely stimulate PKC activity and induce a variety of cellular responses (26, 41). On the other hand, long-term exposure to phorbol ester at a concentration of 1 μM has been shown to downregulate the expression and the activity of PKC in Caco-2 cells and also trigger a number of cellular responses (24). Previous studies from our laboratory (1) have shown that 24-h exposure to PMA increased expression and function of NHE3 but not NHE2 and NHE1 in Caco-2 cells. Therefore, we examined a possible regulation of butyrate uptake by chronic exposure of Caco-2 cells to PMA. Our data showed that butyrate uptake was stimulated in response to long-term PMA exposure in Caco-2 cells. This stimulation in the uptake appears to be via an increase in the Vmax of the carrier-mediated transport process with no alteration in the Km for butyrate. One possibility is that the stimulation in butyrate uptake in response to PMA may be a secondary effect of the increase in NHE3 activity. In this proposed scenario, NHE3 will move more protons out of the cells and, therefore, could decrease the pH in a microdomain around the outer cell membrane. This potential reduction in the pH could then increase the protonation of butyrate in the microdomain around the outer cell membrane and thus increase the rate of diffusion for this short-chain fatty acid. Increase in radioactivity associated with cells could then represent an increase in diffusion of butyrate into cells rather than an increase in function of a carrier-mediated process. However, results of the competition experiment with the analog l-lactate (Fig. 5) rule out this possibility and emphasize the involvement of a carrier-mediated process in upregulation of butyrate transport in response to long-term exposure to PMA in Caco-2 cells. Moreover, attenuation of PMA-stimulated butyrate uptake with an excess of the weak acid l- but not d-lactate further indicates that inhibition with lactate was due to a specific competition with butyrate on the same binding site on a transporter but not due to a decrease in the intracellular pH by acidification by lactate. Based on previous studies of Tamai et al. (43) and Oghihara et al. (33), this stereoselective inhibition of the PMA-stimulated butyrate uptake in Caco-2 cells further suggests involvement of MCT-1. A noteworthy observation is that [14C]butyrate uptake was almost completely inhibited in the presence of 10-fold excess of unlabeled butyrate (data not shown), whereas it was only partially inhibited in the presence of 10-fold excess of l-lactate (Fig. 4). A similar observation was noted in the studies of Buyse et al. (7) in the Caco2-BBE cell line with regard to the partial inhibition of butyrate uptake by MCT-1 inhibitor α-cyano-3-hydroxycinnamate. Poor inhibition of butyrate uptake by MCT-1 inhibitors and substrates was attributed to the possible presence of other transporter(s) in addition to MCTs that may be involved in butyrate transport in Caco-2 cells (7).

As described above, previous studies (24) have shown that long-term exposure to PMA significantly reduced PKC function and expression. The PKC-mediated signal transduction pathway has been shown to be important in the regulation of intestinal ion transport (1, 40, 47). Additionally, previous studies (1) from our laboratory showed that PMA-stimulated NHE-3 function and expression in Caco-2 cells occurred via downregulation of PKCα. In this regard, both PKC and butyrate have been implicated in the regulation of other important cellular processes in colonic epithelial cells such as proliferation, differentiation, apoptosis, and tumorigenesis (2, 6, 13, 17, 21, 31, 45). Therefore, it is plausible to hypothesize that the effect of PMA on butyrate uptake occurs via a PKC-mediated pathway. In a recent study, Cuff et al. (11) showed that butyrate stimulated the function and expression of MCT-1 in the AA/C1 colonic epithelial cell line. Moreover, a previous study by Rickard et al. (36) demonstrated that chronic exposure of colonic cell lines LIM 1215 and Caco-2 to butyrate resulted in downregulation of PKCα. In this regard, both PKC and butyrate have been implicated in the regulation of other important cellular processes in colonic epithelial cells such as proliferation, differentiation, apoptosis, and tumorigenesis (2, 6, 13, 17, 21, 31, 45). Therefore, this hypothesis is plausible that the effect of PMA on butyrate uptake occurs via a PKC-mediated pathway. In a recent study, Cuff et al. (11) showed that butyrate stimulated the function and expression of MCT-1 in the AA/C1 colonic epithelial cell line. Moreover, a previous study by Rickard et al. (36) demonstrated that chronic exposure of colonic cell lines LIM 1215 and Caco-2 to butyrate resulted in downregulation of PKCα, suggesting that some of the biological effects of butyrate may be mediated via a reduction in PKC activity. Because our present studies also demonstrated that butyrate uptake was increased in response to chronic exposure of Caco-2 cells to butyrate, it is logical to propose that the stimulation of MCT-1 expression by butyrate may occur via the downregulation of PKC. Further studies are needed to test this hypothesis. On the other hand, a recent study from our laboratory (29) demonstrated that PMA stimulates NHE2 promoter activity in Caco2-BBE cells in a PKC-
independent manner. Thus it is possible that the effects of PMA on butyrate uptake could also occur via a PKC-independent pathway.

We have also demonstrated that long-term exposure to PMA stimulated MCT-1 expression in parallel to the increase in the $V_{\text{max}}$ of butyrate uptake. However, the MCT-1 protein level was increased by approximately fivefold (Fig. 6), whereas the $V_{\text{max}}$ of butyrate uptake was only increased by $\sim 32\%$ in response to long-term treatment with PMA. One possibility for this observation may be that not all of the MCT-1 protein was targeted to the apical membrane of Caco-2 cells. In this regard, recent studies (7) demonstrated the increase in MCT-1 expression by long-term exposure to leptin and showed that the increase in its function is dependent on the presence of CD147 chaperone protein in Caco2-BBE cells. However, it is not clear from our studies whether a chaperone protein(s) such as CD147 is also involved in the regulation of MCT-1 function and expression in response to long-term PMA exposure in Caco-2 cells. Future studies will focus on a possible involvement of chaperone proteins in PMA-stimulated butyrate uptake in Caco-2 cells.

In summary, the present studies demonstrate that 24-h incubation with PMA but not hydrocortisone stimulated butyrate uptake in Caco-2 cells via upregulation of MCT-1 expression. We have recently cloned the $\delta$ regulator/promoter region of the human MCT-1 gene (15). Future studies should focus on identifying the detailed mechanism(s) of the signaling pathway and the regulatory elements in human MCT-1 promoter that are responsible for this effect.

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