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Induction of Heat Labile Alkaline Phosphatase by Butyrate in Differentiating Endometrial Cells

Honoree Fleming, Michael Begley, Thomas Campi, Rebekah Condon, Katherine Dobyns, Jonathon McDonagh, and Stephen Wallace

Department of Chemistry and Biochemistry, Middlebury College, Middlebury, Vermont 05753 (H.F., M.B., R.C., J.M.); Cornell School of Veterinary Medicine, Ithaca, New York 14853(T.C., K.D.); Stanford School of Medicine, Palo Alto, California 94304(S.W.); Mitsubishi Specialty and Inorganic Chemicals Division, New York, NY 10022

Abstract The addition of 2 mM sodium butyrate to monolayers enhances differentiation of Ishikawa endometrial cells. Cells from this cell line have been shown to enlarge and lift off the dish into dome structures over a period of 24–48 h in response to a factor in fetal bovine serum (FBS) [Fleming, 1995 J Cell Biochem in press]. When butyrate is added to monolayers, together with FBS, three- to fourfold higher numbers of differentiated structures, domes and predomes, can be counted. It had previously been shown [Holinka et al., 1986b] that estradiol induces heat stable placental alkaline phosphatase in Ishikawa cells. The addition of butyrate, on the other hand, results in a significant increase in levels of a heat labile alkaline phosphatase isozyme. The heat labile isozyme is also increased to some extent in cells stimulated to differentiate in response to FBS in the absence of butyrate. Differential inhibition by homoarginine and phenylalanine indicates that butyrate is inducing the liver-bone kidney isozyme that is found in endometrial glands in vivo.

Key words: alkaline phosphatase, Ishikawa endometrial cells, butyrate, differentiation

We have shown that human endometrial cells from the Ishikawa line are capable of differentiating, in response to a protein in fetal calf serum, into elevated multicellular structures. Regions of epithelial cells in monolayer culture become enlarged and lift off the dish, forming fluid-filled domes; cells occasionally bud out from the surface of the dome, extending into elongated structures that resemble inverted glands [Fleming, 1995]. Some of the morphological changes we observed in Ishikawa cells during differentiation, including the initial cell enlargement and the formation of two to four cell predome structures, have not been described in other cell lines. Nevertheless, mature domes, visible in the Ishikawa monolayers 24 h after the addition of serum factor, look like those that form in a number of other human cell lines as reviewed by Rodriguez-Boulan and Nelson [1989].

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Address reprint requests to Honoree Fleming, Department of Chemistry and Biochemistry, Middlebury College, Middlebury, VT 05753.

Different agents have been identified as being able to bring about dome formation in intestinal cells [Lever, 1979] and in other cell lines [Rodriguez-Boulan and Nelson, 1989]. In Ishikawa cells, the agent appears to be a serum factor that behaves as if it is, or is associated with, a macromolecular complex larger than 300 kD. The sex steroid progesterone, responsible for endometrial differentiation in vivo, measurably enhances dome formation in vitro [Fleming, 1995]. In surveying other small biomolecules for an effect on differentiation, we tested the four carbon fatty acid butyrate which can affect differentiation in a variety of different cell types including colon, rectal, blood, and even nerve cell lines [Kruh, 1982].

A second and perhaps related observation about butyrate, initially made more than 20 years ago [Wright, 1973], is that millimolar concentrations are able to inhibit cell proliferation without necessarily causing cell death. This effect has been observed in almost every cell line that has been tested. A third very common effect of butyrate in cultured cells is that it induces alkaline phosphatase. Millimolar concentra-

tions of butyrate have actually only been found in vivo in the intestine and colon, where the fatty acid is thought to accumulate as a result of bacterial metabolism. Nevertheless, cells other than intestinal cells respond to butyrate in vitro, making it a potentially useful tool for studying the process of differentiation. We have found that butyrate affects proliferation and differentiation of the Ishikawa cells and that it causes nearly tenfold increases in activity of a heat labile alkaline phosphatase (ALP) isozyme.

MATERIALS AND METHODS

Ishikawa cells were cultured in phenol-red–free Minimum Essential Medium (MEM) supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and .25 μg/ml amphotericin B (Gibco, Grand Island, NY). The cells were obtained from Dr. Erlio Gurpide at Mt. Sinai Hospital in New York and were originally derived from an endometrial adenocarcinoma line developed by Nishida et al. [1985], who demonstrated the presence of receptors for both estradiol and progesterone. Holinka and his colleagues showed that these cells are responsive to estradiol with regard to proliferation [1986a] and as measured by the induction of alkaline phosphatase [1986b].

Experiments to quantitate differentiation were performed with cells seeded at an approximate density of 5×10^5 cells/cm^2, grown for approximately 3–4 days in 5% fetal bovine serum (FBS) and then transferred to medium containing 1% FBS. Assays for dome formation were done in confluent cultures, although differentiation has been observed in nonconfluent cultures. Cultures left in MEM with 1% FBS can survive for an additional 3–5 days with little proliferation. These minimum levels of FBS keep the cells alive but stimulate little or no dome formation. A 100× solution of sodium butyrate was added to the experimental cells to achieve the final concentration.

Differentiation starts with dramatic changes in individual cells or clusters of cells; cells detach from the dish and become elevated in predome structures which then develop into multicellular structures that look like domes with fluid trapped beneath them. This process requires approximately 24 h. Differentiated structures are easily quantified using an Olympus inverted stage microscope at a power of 40×. Experiments were done in multiwell dishes containing 48 wells or in 35 mm dishes. Differentiated structures, domes and predomes, were always counted over the entire area of the dish or the well since domes sometimes form in clusters. The numbers of differentiated structures are reported, together with the area surveyed.

Assay of Relative Alkaline Phosphatase Activity

Alkaline phosphatase assays were done in 35 mm dishes. At the time of assay (typically after 24 and 48 h incubation in experimental media), medium was removed from each plate. Plates were rinsed in 2 volumes of PBS. Excess PBS was removed by blotting, and rinsed plates were frozen at −20°C for at least 20 min to lyse cells. Prior to the assay, 0.9 mL of DE solution (21.028 g diethanolamine (Sigma, St. Louis, MO), 6.3 mg $MgCl_2$ (Sigma) in 200 mL of H_2O brought to a pH of 9.83) was added to each dish for 15–30 min to completely disrupt the cells and solubilize membrane components, including the ALP enzyme. This extract was added to a 1.5 mL screw-cap eppendorf tubes along with 0.3 mL of wash. After mixing, eppendorf tubes were microfuged for 4 min at room temperature to pellet insoluble cell debris. The supernatant (1.0 ML) was transferred to cuvettes and 60 μl of 18 mM p-nitrophenyl phosphate substrate was added directly to the cuvettes. Three consecutive readings were taken at 60 s intervals, and ALP activity levels were computed on the basis of the change in O.D. of p-nitrophenyl phosphate as a function of time. Each assay was done for DE extract from three separate dishes, and the means are reported. A fourth dish was trypsinized and resuspended in 1 ml of PBS and counted. ALP levels normalized for the number of cells is reported in Figure 3, and absolute ALP levels are reported in Figure 4. ALP activity was measured in the cell monolayer, not in the medium, so the increases measured were not due to increased release of ALP into the medium.

Characterizing Butyrate-Induced ALP Isozyme

In order to characterize the butyrate-induced ALP isozyme in comparison with the E2-induced ALP isozyme, inhibitor studies were done under the same conditions used by Holinka et al. [1986b] as adapted from Fishman [1974]. To test for heat inactivation, enzyme solutions in eppendorf tubes were exposed to 56°C for 15 min in a VWR heating block. Afterwards, eppendorf tubes were removed to an ice bath for 5 min and then allowed to equilibrate to room temperature prior to being assayed as already described. Additionally, ALP activity was tested in the presence of two different amino acid inhibitors.

Either 5 mM phenylalanine or 8 mM homoarginine was
added to solutions of isozymes (human placental ALP, bovine intestinal ALP, or bovine liver bone kidney ALP [Sigma]) or to cell extracts just prior to adding the substrate p-nitrophenyl phosphate. The assay was done as already described.

RESULTS

Butyrate is able to enhance fetal bovine serum–dependent dome formation in Ishikawa cells (Fig. 1). The number of differentiated structures formed in endometrial cell monolayers, counted after 24 h, increases with the addition of increasing concentrations of FBS to cells maintained in medium containing 1% FBS. At least three- to fourfold more domes are counted when 2 mM butyrate is added together with 5%, 10%, or 20% FBS. Sometimes, as in this experiment, butyrate is even able to bring about dome formation in the presence of only 1% FBS, an amount which is usually too low to effect differentiation.

Figure 2 demonstrates the antiproliferative effects of butyrate in Ishikawa cells. Cells were plated with a starting density of 2×10^5 cells per dish on day 0. On days 2 and 4, 2 mM sodium butyrate was added to some of the dishes. Within 1 day, butyrate caused a cessation of cell proliferation. The effect was reversible: proliferation resumed when cells, treated with butyrate on day 2, were transferred to fresh medium without butyrate on day 4.

In addition to enhancing differentiation and inhibiting proliferation, butyrate induces increased levels of alkaline phosphatase enzymatic activity. Figure 3 shows that the addition of 2 mM butyrate to cultures maintained in 1% FBS resulted in a more than sevenfold increase in ALP activity measured as a function of cell number at 24 and at 48 h after the addition of butyrate, with no evidence of differentiation. The addition of 15% FBS resulted in an approximately twofold elevation in ALP and some differentiation, a range of 20–30 domes in each of three dishes. The addition of 15% FBS and butyrate elevated ALP to levels tenfold higher than

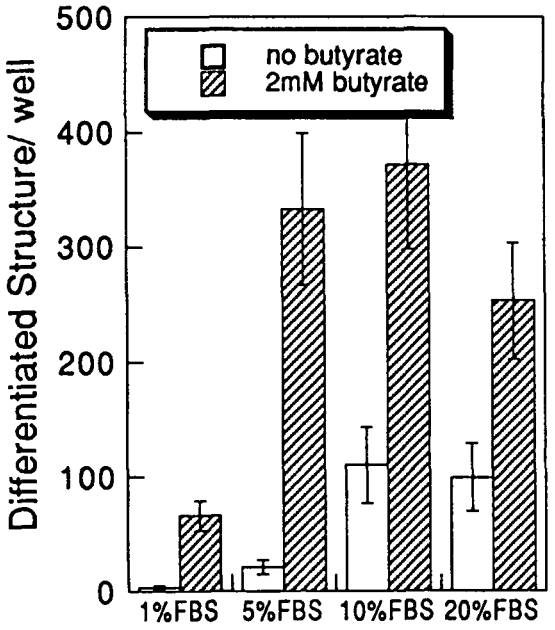

Fig. 1. Effects of 2 mM butyrate on dome formation. Different concentrations of FBS were added to confluent monolayers of Ishikawa cells grown to confluence in dishes containing 48 wells, with or without the addition of 2 mM butyrate. The numbers of predomes and domes are compared on day 1 when the number of differentiated structures is at its highest. These values represent the means ± SE for three wells for each condition.

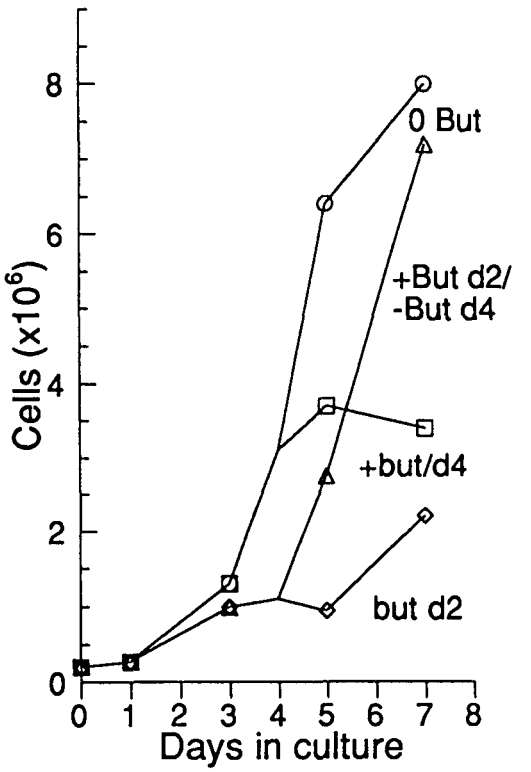

Fig. 2. Effects of 2 mM butyrate on cell proliferation. Cells were grown in medium containing 5% FBS in 35 mm dishes. On days 2 and 4, 2 mM butyrate was added to dishes as indicated. On day 4, the medium was changed, and some of the dishes to which 2 mM butyrate was added on day 2 were transferred to medium without butyrate. Cell counts done on successive days showed that butyrate stopped proliferation, an effect that could be reversed. The values represent means of two dishes; three separate counts were done for each dish, and individual values were within 10% of the mean.

the control, with the formation of from 200–300 domes in each of three dishes 24 h after the additions were made. These ALP levels were slightly, but not significantly, higher than the values found for butyrate and 1% FBS.

The ALP activity induced by the addition of butyrate, or in the presence of 15% FBS, was tested to determine whether it is heat stable or heat labile isozyme. Placental and germ cell ALP isozymes are stable to heat inactivation at 56°C, while two other isozymes, liver/bone/kidney (LBK) and intestinal, are readily heat inactivated. When the low levels of ALP in control cells were tested for heat lability by incubating the enzyme extract at 56°C for 15 min, it was found that approximately 50% of the enzyme was heat labile. As Figure 4 shows, almost all of the increased ALP activity induced by the addition of butyrate, 15% FBS, or butyrate and 15% FBS could be inactivated by incubating cell extracts at 56°C for 15 min, indicating that the ALP induced a nonplacental ALP isozyme as opposed to the placental isozyme induced by estradiol in these cells.

In an effort to further characterize this ALP isozyme, human placental ALP, bovine intestinal ALP, and bovine LBK ALP were assayed following incubation at 56°C or in the presence of homoarginine or phenylalanine (Table I). The activity profiles found for these isozymes can be distinguished by different reactions to these three inhibitory conditions, as was reported by Fishman [1974]. The ALP isozyme induced by butyrate was found to be heat labile, 80% inhibited by homoarginine, and only moderately inhibited by phenylalanine. These are the characteristics of the LBK ALP isozyme. This is the variety of ALP that has been measured in freshly isolated human endometrial glands in both the proliferative and the secretory phase [Holinka and Gurpide, 1981].

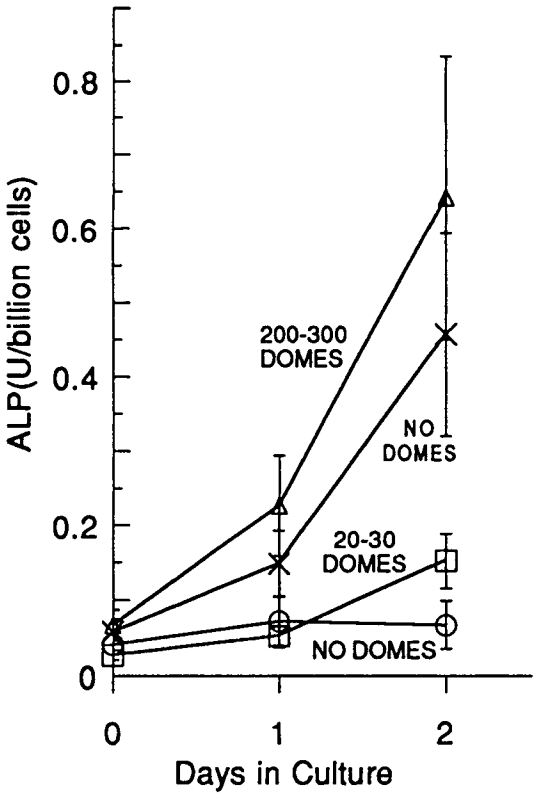

Fig. 3. Effects of 2 mM butyrate on induction of alkaline phosphatase. Cells were grown to confluence in 35 mm dishes. The following conditions were tested for their effects on ALP activity: medium containing 1% FBS (o—o), 1% FBS + 2 mM butyrate (x—x), 15% FBS (□—□), and 15% FBS + 2 mM butyrate (△—△). Under these conditions, maximal dome formation occurs approximately 24–30 h after the addition of FBS or FBS + 2 mM butyrate. The number of domes had subsided by the second day of culture. No domes were found in the cultures containing 1% FBS ± 2 mM butyrate. The ALP values represent the means ± SE for three dishes for each condition.

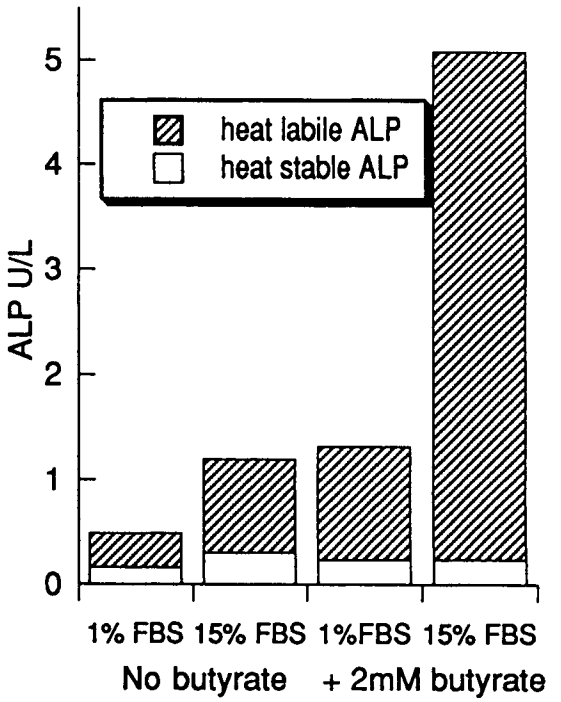

Fig. 4. Heat inactivation of alkaline phosphatase activity induced by butyrate. Alkaline phosphatase induced in response to FBS, to butyrate, and to both together was tested for heat lability by incubating the extract at 56°C for 15 min, conditions that do not inactivate placental ALP.

TABLE I. Inhibition of ALP Isozymes by Heat Inactivation and Amino Acid Inhibitors*

	56°C/ 15 min (%)	8 mM homo-arginine (%)	5 mM phenyl-alanine (%)
Inhibitor effects on ALP isozymes			
Human placental	0	10	80
Bovine liver-bone-kidney	95	83	38
Bovine intestinal	95	0	29
Experimental results			
Ishikawa[a] (control)	51	24	44
Ishikawa + 15% FBS + 2 mM butyrate	95	80	54

*Each of three different ALP isozymes was tested for the effects of various inhibitors. At the same time ALP induced in Ishikawa cells by butyrate was tested for inhibitor effects. Each of the assays was done in triplicate, and the average of the percent of inhibition is shown.

DISCUSSION

Cells undergoing differentiation or partial differentiation in monolayer culture provide a reasonable in vitro system to investigate this important developmental process. Evidence for differentiation includes microscopically visible morphological changes in monolayer cells, histochemical changes in cell membrane reactivity, and/or changes in enzymatic activities in cell extracts. Unique differentiating agents have been found for particular cell types, but it has also been observed that some chemical agents enhance differentiation in more than one cell type. The vitamin A derivative retinoic acid reviewed by De Luca [1991] and the secondary messenger cAMP reviewed by Roesler et al. [1988] are two examples of such differentiating agents. One other biochemical that has unexpectedly emerged as a differentiating agent for a number of different cell types is the four carbon fatty acid butyrate [Prasad, 1980; Kruh, 1982].

Butyrate has been shown to slow proliferation and elicit differentiation in a variety of different cell lines [Ginsburg et al., 1973; Prasad and Sinha, 1976; Dulbecco et al., 1980], including erythroleukemia [Leder and Leder, 1975], colon [Kim et al., 1980], rectal [Tsao et al., 1982], breast [Abe and Kufe, 1982], intestinal [Joshi et al., 1985], prostatic [Reese et al., 1985], salivary [Azuma et al., 1986], fat [Toscani et al., 1988], liver [Gladhaug et al., 1988], ovarian [Langdon et al., 1988] and pancreatic cells [Mullins et al., 1991]. The changes that signal differentiation are quite specific for the cell type being tested; nevertheless, all of these different changes are elicited by the same small fatty acid alone or in concert with another inducer. In this paper we report that millimolar concentrations of butyrate are able to enhance differentiation and dome formation in human endometrial cells when added together with FBS, previously shown to contain a factor essential for dome differentiation [Fleming, 1995]. Similarly, butyrate enhancement of differentiation has been demonstrated for the dexamethasone- and insulin-dependent induction of adipocyte differentiation [Toscani et al., 1990] and for the 1,25-dihydroxyvitamin D3 induction of colon cell differentiation [Tanaka et al., 1990]. Combined inducer effects have also been described for butyrate and a Ca^{++} ionophore A23187 in HT29 cells [Higgins and Lipkin, 1994] and for butyrate and transforming growth factor β in keratinocytes [Wang et al., 1992].

Given the diversity of differentiated states resulting from butyrate addition, with or without another factor, it is not surprising that this fatty acid has been reported to induce numerous proteins in various cell lines. These proteins include enzymes [Prasad and Sinha, 1976], particularly cell surface enzymes [Griffin et al., 1974], receptors for epidermal growth factor and transforming growth factor [Sheikh et al., 1994], and hormones such as glucagon and insulin [Philippe et al., 1987; Bartholomeusz et al., 1989]. Alkaline phosphatase is reported to be induced by butyrate more often than any other enzymatic activity, but it is not a single gene that is affected. Each of the four different ALP isozymes has been shown to be induced by butyrate in different cell lines. Heat stable placental alkaline phosphatase is induced in several rectal carcinoma cells [Herz et al., 1981; Tsao et al., 1983; Heruth et al., 1993] and in ovarian cells [Yabushita and Sartorelli, 1993]. Another heat stable ALP called germ cell alkaline phosphatase (GCAP) is induced by butyrate in choriocarcinoma [Ito and Chou, 1984; Povinelli et al., 1992] and in the colon LS174T cells [Gum et al., 1987]. It has been suggested that these two heat stable enzymes are oncofoetal proteins [Fishman, 1985]. The two other ALP isozymes that can be induced by buturate are heat labile and assumed to be more characteristic of the differentiated state. Liver-bone-kidney (LBK) ALP is induced by butyrate in the colon cell line SW620 [Herz and Halwer, 1990] and in murine lym-

phoma cells [Harb et al., 1991]. Intestinal ALP is reported to be induced in yet another colon carcinoma cell line, HT29 [Herz et al., 1981]. These inductions of ALP coincide with a significant increase in the doubling time of the cells and in the appearance of features characteristic of differentiation.

Our results in Ishikawa cells clearly demonstrate that butyrate induces a heat labile ALP *different from* the heat stable placental alkaline phosphatase induced by estradiol [Holinka et al., 1986b; Albert et al., 1990]. The existence of two ALP isozymes in Ishikawa cells was initially demonstrated by results of Albert et al. [1990]. The fact that this heat labile isozyme is 80% inhibited by homoarginine further indicates that the isozyme is probably identical to the LBK enzyme previously shown to be the dominant isozyme in human endometrial glands isolated from either the proliferative or the secretory tissue [Holinka et al., 1980].

Other cell lines reported to contain more than one ALP include Capan-1 pancreatic cells with placental and LBK ALP [Fanjul et al., 1991] and choriocarcinoma cells with both kinds of heat stable ALP [Watanabe et al., 1989]. As in the Ishikawa cells, butyrate is usually found to preferentially induce only one of the ALP enzymes [Herz and Halwer, 1990; Povinelli et al., 1992]. What has not necessarily been seen before is that in the Ishikawa cells two different ALP isozymes can be induced by two different agents: the mitogen E2 induces a placental, perhaps oncofoetal heat stable ALP isozyme, while butyrate induces a heat labile ALP characteristic of the differentiating glands in vivo.

Butyrate-induced ALP levels began to increase within 24 h of its addition to confluent monolayers, at the same time that numerous domes appeared. Peak numbers of differentiated structures, under the conditions of this experiment, appear on day 1. ALP enzyme levels continue to increase on day 2 even as the number of differentiated structures declines. Gum and his colleagues [1987] found a similar pattern in butyrate induction of heat stable ALP in the intestinal line LS174T, except that there was a lag time of 48 h prior to the increase in ALP levels and dome formation; thereafter, both activities increased for 48 h. When the number of domes began to decline, at 96 h, ALP activity continued to increase for another 90 h.

These results demonstrate that while induction of ALP activity may accompany differentiation, it continues even when the differentiated state is no longer obvious. In fact, although prominent alkaline phosphatase activity has been found associated with apical surfaces of domes in a renal line [Rabito et al., 1984] and in the pancreatic Capan 1 cells [Fanjul et al., 1991], the activity is not confined to the domes. Butyrate can effect an increase in ALP activity even when dome formation is not obvious. At the same time, our results demonstrate FBS-induced differentiation results in ALP induction even in the absence of butyrate, indicating that ALP induction may accompany differentiation, but the induction also occurs even when differentiation does not.

In addition to its effects on differentiation and alkaline phosphatase induction, we have also found that butyrate inhibits cell proliferation of the Ishikawa cells. This butyrate effect has been found in most of the cell lines tested. In early studies of HeLa cells [Fallon and Cox, 1979], fibroblasts [Wintersberger et al., 1983], and intestinal cells [Darzynkiewicz et al., 1981] and in more recent studies of adipocytes [Toscani et al., 1988] and rat insulinoma cells [Karlsen et al., 1991], it has been reported that cells are stopped in the G_1 phase of the cell cycle. It is possible that butyrate's ability to pause cells in a certain part of the cycle allows the cells to initiate differentiation with resulting changes in protein expression and morphology [Scott et al., 1982]. Such a coupling of growth arrest and differentiation was recently described for colonic epithelium [Higgins and Lipkin, 1994].

Interest in drugs that have antiproliferative and differentiating effects on cells has increased among researchers studying cancer. Butyrate and other differentiating agents are being considered as potential treatment for melanoma and hematopoietic and solid neoplasms [Sartorelli, 1985; Reiss et al., 1987]. The wide-ranging effects of butyrate on different cell types suggest some common mechanism at work in the process of differentiation, a mechanism whose activation may involve inhibition of cell division. It is not known by what mechanism butyrate inhibits cell cycling. It was reported more than 15 years ago that the addition of butyrate can alter the extent of histone acetylation in cultured cells [Riggs et al., 1977; Candido et al., 1978; Sealy and Chalkley, 1978], and it has been shown that butyrate can affect phosphorylation of two histones: H-1 and H-2 [D'Anna et al., 1980;

Boffa et al., 1981]. The suggestion has been made that these activities of butyrate, resulting in alteration in DNA structure, might be responsible for changes in cell cycle and differentiation state, but there is very little evidence as to how this would occur. Possible butyrate effects on transcription, posttranscriptional processing, and translation are also mechanisms that are being investigated.

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