SODIUM BUTYRATE BLOCKS HELA CELLS PREFERENTIALLY IN EARLY $G_1$ PHASE OF THE CELL CYCLE

SHAOBAI XUE* AND POTU N. RAO

Department of Developmental Therapeutics, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, Texas 77030, U.S.A.

SUMMARY

The effects of sodium butyrate on the cell-cycle traverse of HeLa cells was re-examined using the kinetic and the premature chromosome condensation methods of cell-cycle analysis. The results of this study indicate that the sodium butyrate effects are dose-dependent and cell-cycle phase-specific. Cells in the early $G_1$ period are the most sensitive to the inhibitory effects of the drug. When HeLa cells in exponential growth were exposed to a 5 mM concentration of butyrate for 48 h, 67% of the cell population was preferentially arrested in the early $G_1$ period as indicated by the condensed morphology of the prematurely condensed chromosomes (PCC). There was no accumulation of cells in the $G_2$ period. The results of this study suggest that hyperacetylation of histones induced by sodium butyrate does not necessarily result in decondensation of chromatin and the butyrate-enhanced protein, which has been reported to be present in butyrate-treated cells, may have a role in the regulation of the transition of cells from early $G_1$ to the subsequent phases of the cell cycle.

INTRODUCTION

The cytostatic effects of sodium butyrate were first observed in human cells in vitro by Pace, Aftonomos, Elliot & Sommer (1967). Since then this compound has been studied extensively and the results of these studies indicate that butyrate has profound effects on the morphology, growth rate, and gene expression in mammalian cells in culture (Wright, 1973; Ginsburg, Salomon, Sreevalson & Freese, 1973; Fishman, Simmons, Brady & Freese, 1974; Henneberry, Fishman & Freese, 1975; Ghosh, Deutsch, Griffin & Cox, 1975; Leder & Leder, 1975; Prasad & Sinha, 1976; Altenburg, Via & Steiner, 1976; Hagopian, Rigs, Swartz & Ingram, 1977; Tralka et al. 1979; Fallon & Cox, 1979; D'Anna, Tobey & Gurley, 1980; Prasad, 1980). Sodium butyrate has been shown to slow down cell-cycle traverse, inhibit DNA synthesis, and block cells in $G_1$ phase of the cell cycle (Hagopian et al. 1977; Fallon & Cox, 1979; D'Anna et al. 1980). The effects of butyrate are dose-dependent and reversible. However, there are some obvious discrepancies between the results from different laboratories. The studies of Hagopian et al. (1979) reveal that in HeLa cells exposed to butyrate (5 mM for 48 h) there is a significant increase in the proportion of cells residing in $G_1$ and $G_2$ phases, but a marked decrease in $S$-phase cells. They have

* Present address: Department of Biology, Beijing Normal University, Beijing, People's Republic of China.
concluded that more than 50% of the butyrate-treated HeLa cells are located in either late G1 or early S phase (Hagopian et al. 1979). In contrast, the studies of D’Anna et al. (1980) indicate that although butyrate slows down the progression of Chinese hamster ovary (CHO) cells in all phases of the cell cycle, it does not arrest them indiscriminately; rather, the cells become arrested predominantly in G1 phase. Furthermore, Darzynkiewicz et al. (1981), using flow cytometric techniques, recently observed that the butyrate-treated L1210 cells are arrested in the early G1 phase of the cell cycle. In view of these discrepancies we have decided to re-examine the effects of butyrate on HeLa cell-cycle traverse using 2 different methods of cell-cycle analysis: a kinetic method and the premature chromosome condensation method. The premature chromosome condensation method is best suited to determining the exact point of cell arrest in the cell cycle. The specific objective of this study was to determine whether HeLa cells in different phases of the cell cycle respond differently to treatment with butyrate and whether the treated cells would be blocked in early or late G1 phase. The results of this study indicate that the inhibitory effects of butyrate are more pronounced on G1 and early S-phase cells and the butyrate-treated cells are preferentially blocked in early G1 phase.

MATERIALS AND METHODS

Cells and cell synchrony

HeLa cells used in this study were grown in Lux plastic dishes as monolayer cultures in McCoy’s medium 5A (GIBCO Santa Clara, Calif.) supplemented with 16% heat-inactivated foetal calf serum and 1% penicillin (10000 units/ml) and streptomycin (10000 µg/ml). These cells have a cell-cycle time of approximately 22 h and a plating efficiency of about 90%. Mitotic cells of 98% purity were obtained by selective detachment after exposure to Colcemid (0.5 µg/ml) from a monolayer culture that was partially synchronized into S phase by a single thymidine (2.5 mM) block of 16 h duration.

Drug treatment

To determine the effective dose, various concentrations (0, 3 and 5 mM) of sodium butyrate were added to cultures in exponential growth that were plated with a known number of cells per dish. Two dishes for each treatment were trypsinized to determine the cell counts at 0, 24, 48 and 72 h of incubation with the drug.

Cell-cycle kinetics

The effects of sodium butyrate on the progression of HeLa cells through the cycle were monitored by the method of Puck & Steffen (1963). In these experiments HeLa cells in exponential growth were plated in a number of 35 mm culture dishes 1 day before the experiment. We began the experiment by replacing the old medium in the dishes with fresh medium containing Colcemid (0.05 µg/ml) and [3H]thymidine (0.05 µCi/ml; sp. act. 6.7 Ci/mmol). We took samples at regular intervals by trypsinizing the cells and depositing them directly on clean slides with a cytocentrifuge. The cells on the slides were then fixed in absolute methanol:glacial acetic acid (3:1) mixture, extracted twice with cold 5% trichloroacetic acid, processed for autoradiography, and scored for labelling and mitotic indices.

Total mitotic index (TMI) = (number of labelled + unlabelled mitoses/all mitotic and interphase cells scored) x 100.

Labelled mitotic index (LMI) = (number of labelled mitoses/all mitotic and interphase cells scored) x 100.
Labelling index (LI) = (number of labelled mitoses + interphases/all mitotic and interphase cells scored) x 100.

Cell fusion

The procedures for cell fusion were essentially the same as those described by Rao & Johnson (1970). This involved the mixing of an equal number of untreated mitotic cells with treated interphase cells in serum-free medium, centrifugation of the mixture, and resuspension of the cell pellet in 0.5 ml of phosphate-buffered saline containing 2000 haemagglutination units of ultraviolet light inactivated Sendai virus. The fusion mixture was placed in 4 °C for 15 min and then incubated in a 37 °C water-bath for 45 min. By this time, the cells had fused and premature chromosome condensation was induced.

To determine where the treated cells were blocked, we applied the method of premature chromosome condensation, which involves fusion between mitotic and interphase cells. Thus the position of an interphase cell in the cell cycle may be determined on the basis of the morphology of its prematurely condensed chromosomes (PCC). For example, G1 cells produce PCC with single chromatids, and G2 cells yield PCC with double chromatids. G1 phase could be subdivided further as early, mid and late, depending on the degree of decondensation of the G2 PCC. Early G1 PCC are short and condensed; late G2 PCC are highly extended, while mid G1 PCC are intermediate between these 2 types (Rao, Wilson & Puck, 1977; Hittelman & Rao, 1978). PCC from S-phase cells appeared pulverized. Cells in exponential growth were exposed to various concentrations (0, 3 and 5 mM) of sodium butyrate, and at 48 h of incubation with the drug, the treated and the untreated control cells were separately fused with synchronized mitotic HeLa cells to induce PCC. Chromosome preparations were made and scored for the frequency of the various types of PCC, i.e. G1, S and G2.

Radioautography

For the determination of labelling index, cells were incubated with [3H]thymidine (0.1 μCi/ml; sp. act. 6.7 Ci/mmol) for 30 min, trypsinized, and deposited on clean slides using a cytocentrifuge. The cells were fixed in a methanol:glacial acetic acid (3:1) mixture for 5 min. For autoradiography, the slides were extracted 3 times (20 min each) in cold (4 °C) 5% trichloroacetic acid to remove the non-specific label and then rinsed in distilled water. Dried slides were dipped in Kodak nuclear emulsion (NTB-2, Eastman Kodak Co., Rochester, N.Y.) diluted 1:3, allowed to dry, and stored in light-tight boxes for 2 days. The slides were processed in D-19 developer for 2 min and in Kodak fixer for 2 min. They were washed and stained with Giemsa.

Results

Drug effects on cell division

The increase in cell number as a function of time and drug concentration is shown in Fig. 1. At a concentration of 5 mM-sodium butyrate, the cell number increased by approximately 30% within the first 24 h of treatment and then reached a plateau. At 3 mM concentration, the growth rate was slower than the control, but it was exponential.

Drug effects on cell-cycle traverse

In order to find out the cell-cycle phase in which the treated cells were arrested, HeLa cells in exponential growth were exposed on 0, 3 and 5 mM-sodium butyrate for 48 h and then fused with synchronized mitotic HeLa cells. The resulting PCC were classified as early G1, mid G1, late G1, S and G2 (Table 1). With increasing dose
there was a significant increase in the frequency of early G₁, PCC. The increased accumulation of cells in the early G₁ period observed in the 5 mM treatment was associated with a corresponding decrease in the frequency of the cells in late G₁, S and G₂. At 3 mM concentration the frequencies of cells in the S and G₂ phases were not significantly different from those in the control.

**Table 1. Estimation of cells in various phases of the cell cycle by the PCC method in control and butyrate-treated cultures**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Early G₁</th>
<th>Mid G₁</th>
<th>Late G₁</th>
<th>S</th>
<th>G₂</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.1</td>
<td>14.9</td>
<td>14.4</td>
<td>35.3</td>
<td>10.2</td>
<td>100</td>
</tr>
<tr>
<td>Sodium butyrate, 3 mM for 48 h</td>
<td>47.9</td>
<td>8.6</td>
<td>4.6</td>
<td>36.2</td>
<td>7.6</td>
<td>100</td>
</tr>
<tr>
<td>Sodium butyrate, 5 mM for 48 h</td>
<td>67.8</td>
<td>5.9</td>
<td>1.2</td>
<td>21.4</td>
<td>3.6</td>
<td>100</td>
</tr>
</tbody>
</table>

To answer the question whether cells in all phases of the cell cycle are equally sensitive to the inhibition by sodium butyrate, we have performed cell-cycle analyses in the control and treated (5 mM) cultures using the method of Puck & Steffan (1963). The results of these experiments (shown in Fig. 2) indicated that all the cells in G₂ at the beginning of the treatment entered mitosis at about the same rate as in the control as evidenced by the slope of the total mitotic index curves. Of the 34% of cells in S phase (initial LI) only 22% (maximum LMI in Fig. 2A) were able to enter mitosis in the treated culture and the duration of G₂ for these cells was prolonged, i.e. approximately 8 h compared with 4.5 h in the control. In other words a third of the S-phase
G_1 arrest of sodium butyrate

population, probably the early S-phase population, failed to reach mitosis and was arrested in late S or G_2 (Fig. 3). In the control (Fig. 2A), the difference, which was 46% between the maximum LI and initial LI, indicates the proportion of cells residing in mitosis and G_1 at the beginning of the experiment. Of this 46% only 18% (the maximum LI of 52% minus the initial LI of 34% shown in Fig. 2B) entered S phase in the butyrate-treated culture (Figs. 2B, 3).

Fig. 2. Cell-cycle analysis of the control (A) and sodium butyrate-treated (B) HeLa cells by the method of Puck & Steffen (1963). HeLa cells in exponential growth were plated in a number of 35 mm plastic culture dishes a day before the experiment. The experiment was begun by adding [3H]thymidine (0.05 μCi/ml; sp. act. 6.7 Ci/mmol) and Colcemid (0.05 μg/ml) to all the dishes. Sodium butyrate (5 mM) was added to half of the dishes while the other half served as controls. Cell samples were taken at regular intervals. O—O, total mitotic index (TMI); •—•, labelled mitotic index (LMI); A—A, labelling index (LI).

Fig. 3 summarizes the data from both the PCC and kinetic methods of cell-cycle analysis. This figure provides an account of the whole cell population in various phases of the cell cycle before and after treatment with 5 mM-sodium butyrate. A majority of those cells (23%) that were in the early G_1 period at the time of treatment failed to proceed any further in the cell cycle as revealed by the PCC data. Late G_1 cells (18%) became arrested in early S, and early S cells (11%) in late S or G_2. Cells that were in late S (22%), G_2 (18%) and mitosis (4%) at the time of the addition of the drug
Fig. 3. A schematic representation of the effects of sodium butyrate (5 mM) treatment on the cell-cycle traverse of HeLa cells based on the data from Fig. 2 and Table 1. EG₁, early G₁. Maximum LI (80%) in Fig. 2A minus maximum LI (54%) in Fig. 2B is equal to 26%, which represents the percentage of G₁ cells that failed to enter S phase as a result of butyrate treatment. LG₁, late G₁. Maximum LI (54%) minus initial LI (36%) in Fig. 2B is equal to 18%, which represents late G₁ cells that could enter S phase after the drug has been added to the culture. ES₁, early S. Initial LI (36%) minus maximum LMI (22%) in Fig. 2B is equal to 14%, which represents the cells that were in S when butyrate was added to the culture but failed to reach mitosis. LS₁, late S. The percentage of cells in late S is equal to the maximum LMI (22%) in Fig. 2B. These cells that were in S phase at the time butyrate was added were able to enter mitosis. G₂, maximum TMI (40%) minus maximum LMI (22%) in Fig. 2B = 18%. These were the cells that were in G₂ at the beginning of the experiment. M, the mitotic index of a random population before the beginning of the experiment was about 4%. The small numbers indicate the percentage of cells residing in each phase of the cell cycle before the treatment. The percentages in parenthesis indicate the fraction of the cell population residing in the phase of the cell cycle after 48 h of treatment. The arrows indicate the extent of cell-cycle progression during treatment. The solid rectangles at the arrowheads indicate a block in cell-cycle progression.

Discussion

The results of this study indicate that the inhibitory effects of sodium butyrate are dose-dependent and cell-cycle phase-specific. At a concentration of 3 mM-butyrate, the growth rate was slowed down but not completely inhibited (Fig. 1). However, at 5 mM concentration, only those cells in late S, G₂ and mitosis were able to complete division (Fig. 2B) and were subsequently blocked in the early G₁ period as indicated by the PCC method of analysis (Table 1). In the light of both the kinetic and the
PCC we conclude that the accumulation of 67% of the treated population in early G1 is the result of the failure of early G1 cells to proceed any further in the cell cycle, and in addition to the arrest of late S, G2 and mitotic cells in early G1 subsequent to completion of one cell division following the addition of the drug to the culture (Fig. 3). During the course of the treatment, a portion of the early G1 cells might have progressed into late G1, those in late G1 into S, and those in early S into late S or G2 (Fig. 3). Thus it appears that cells in early G1 phase are the most sensitive to treatment with butyrate even though cells in other parts of the cell cycle are also affected to some extent. Unlike Hagopian et al. (1977) we did not observe any significant G2 arrest. Our observation of early G1 arrest of HeLa cells by sodium butyrate is in agreement with the report of Darzynkiewicz et al. (1981).

Treatment with sodium butyrate has been reported to cause extensive, though reversible, acetylation of histones in HeLa cells after 24 h of treatment at 5 mM concentration (Riggs, Whittaker, Neumann & Ingram, 1977). The presence of histones in highly acetylated form is presumed to be due to the inhibitory effects of sodium butyrate on the activity of histone deacetylases (Hagopian et al. 1977). In Physarum polycephalum the overall proportion of highly acetylated H4 histone is at its minimum (13.5%) in early prophase and at its maximum (33.6%) in mid-S phase (Chahal, Mathews & Bradbury, 1980). Thus acetylation of H4 histone is inversely correlated to H1 histone phosphorylation and chromosome condensation. In view of these data one would expect the butyrate-arrested cells to exhibit PCC with diffused morphology. In fact the opposite, i.e. highly condensed G1 PCC, was found. From another point of view the arrest of HeLa cells in the early G1 period by sodium butyrate was unexpected. Under suboptimal conditions of growth (i.e. high cell density, serum starvation, deprivation of leucine or isoleucine, and inhibition of polyamine biosynthesis) normal diploid fibroblasts are arrested in G0 or early G1 while transformed cells are blocked in late G1, as indicated by the diffused morphology of the PCC of the treated cells (Hittelman & Rao, 1978; Sunkara, Paragac, Nishioka & Rao, 1979).

Using the PCC method we have previously demonstrated that there is a progressive decondensation of chromatin during the G1 period, reaching the most diffused or decondensed state by the beginning of S phase (Rao et al. 1977; Hittelman & Rao, 1978). If hyperacetylation is correlated with decondensed chromatin we would expect the butyrate-treated cells to be blocked in late G1 period. The fact that 67% of the population is blocked in early G1 suggests that the treatment with sodium butyrate in some way prevents the progression of HeLa cells from early G1 into late G1 when the chromatin becomes gradually decondensed. These results suggest that hyperacetylation of histones does not necessarily result in decondensation of chromatin. In addition to changes in histone modification in CHO cells treated with sodium butyrate, some quantitative changes in 2 proteins, which coincidentally migrate into the H3 region of one- and two-dimensional gels, have been reported (D’Anna et al. 1980). According to these authors, one of these proteins, called butyrate-enhanced protein (BEP), increased in the butyrate-treated cells in a dose-dependent manner. D’Anna et al. (1980) have also observed that the BEP/H1 ratio is greater at higher
butyrate concentrations when cell growth has virtually ceased. Hence it is possible
that the butyrate-enhanced protein may have a role in blocking the progression of
cells from early \( G_1 \) to the subsequent phases in butyrate-treated cultures. This
hypothesis remains to be tested.

This investigation was supported in part by research grants CA11520, CA-23878, CA27544
and CA-5831 from the National Cancer Institute, DHHS, and grant CH-152 from the American
Cancer Society. S. Xue is a visiting scholar to the United States whose work here was made
possible through the National Academy of Sciences Committee on Scholarly Communication
with the People's Republic of China.

REFERENCES

sarcoma virus-transformed cells by sodium butyrate. Expl Cell Res. 102, 223-231.


sodium butyrate in Chinese hamster cells: Cell-cycle progression, inner histone acetylation,
histone H1 dephosphorylation, and induction of an H1 like protein. Biochemistry 19,
2656-2671.


FALLON, R. J. & COX, R. P. (1979). Cell cycle analysis of sodium butyrate and hydroxyurea,
inducers of ectopic hormone production in HeLa cells. J. cell. Physiol. 100, 251-262.


morphological modulation of HeLa cells in monolayer culture by dibutryl cyclic AMP,
butyrate and their analogs. J. cell Physiol. 86, 663-672.

GINSBURG, E., SALOMON, D., SREEVALSON, T. & FRESE, E. (1973). Growth inhibition and
U.S.A. 70, 2457-2461.

on DNA synthesis in chick fibroblasts and HeLa cells. Cell 12, 855-860.

HENDRICKER, R. C., FISHMAN, P. H. & FRESE, E. (1975). Morphological changes in cultured

HITTELMAN, W. N. & RAO, P. N. (1978). Mapping \( G_1 \) phase by the structural morphology of
the prematurely condensed chromosomes. J. cell. Physiol. 95, 333-342.

LEDER, A. & LEBER, P. (1975). Butyric acid, a potent inducer of erythroid differentiation in
cultured erythroleukemic cells. Cell. 5, 319-322.

effects of the sodium salts of certain monocarboxylic acids on established cell lines. Can.
J. Biochem. 45, 81-88.

PRASAD, K. N. (1980). Butyric acid: A small fatty acid with diverse biological functions. Life
Sci. 27, 1351-1358.

PRASAD, K. N. & SINHA, P. K. (1976). Effect of sodium butyrate on mammalian cells in culture:
A review. In vitro 12, 125-132.

localizing metabolic events within the life cycle, and its application to the action of Colcemid


cell cycle analysis. J. cell. Physiol. 91, 131-142.
G1 arrest of sodium butyrate


(Received 19 March 1981)