CELL-CYCLE-DEPENDENT EFFECTS OF SODIUM-\textit{n}-BUTYRATE IN \textit{PHYSARUM POLYCEPHALUM}

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SUMMARY

Sodium-\textit{n}-butyrate affects the length of the mitotic cycle of \textit{Physarum polycephalum}. Application during \textit{S}- or early \textit{G2}-period results in a delay of the subsequent mitosis, whereas application later in the cycle has no delaying effect. Interestingly, the second mitotic cycle after application is considerably shortened when butyrate has been administered during \textit{S}- or early \textit{G2}-period of the preceding cycle. In comparison, other homologous short-chain fatty acids were tested; the retarding effect on mitosis increases with the number of carbon atoms, although only butyrate can shorten the second mitotic cycle. It is shown that butyrate causes an immediate depression of synthesis of DNA, RNA and protein. After a certain time-interval the plasmodium overcomes the butyrate block. DNA synthesis is fully recovered and the inhibition of RNA and protein synthesis is even overcompensated until the next mitosis, as reflected by elevated levels of RNA and protein.

INTRODUCTION

There are numerous reports in the literature, that sodium-\textit{n}-butyrate has a variety of effects on several cell types (for a recent review, see Kruh, 1982). Among the manifold and often tissue-specific effects there are two that occur in almost all cell lines: arrest of proliferation and inhibition of histone deacetylase.

Inhibition of histone deacetylase leads to hyperacetylation of histones H3 and H4 (Riggs, Whittaker, Neumann & Ingram, 1977; Vidali, Boffa, Mann & Allfrey, 1978). This fact is of particular importance, since a connection between histone acetylation and gene expression has been frequently discussed and chromatin containing highly acetylated histones is correlated with the active site of chromatin (Gorovsky, Pleger, Keevert & Johnmann, 1973; Leder & Leder, 1975; Marushige, 1976; Oberhauser, Csordas, Puschendorf & Grunicke, 1978; Simpson, 1978; Vidali, Boffa, Bradbury & Allfrey, 1978; Dobson & Ingram, 1980).

As already mentioned, sodium-\textit{n}-butyrate is an inhibitor of cell growth as demonstrated in various systems (Wright, 1973; Ginsburg, Salomon, Sreevalsa & Freese, 1973; Hagopian, Riggs, Swartz & Ingram, 1977; Leibovitch & Kruh, 1979). The arrest of proliferation was always found to be fully reversible.

We set out to study the effect of sodium-\textit{n}-butyrate and homologous short-chain fatty acids in the myxomycete \textit{Physarum polycephalum}. \textit{Physarum} has attracted considerable interest as a model system for studies of cell-cycle-dependent events, since
nuclear division occurs spontaneously and synchronously. The nuclear division cycle of *Physarum* macroplasmodia consists of the S-phase (3 h duration), which immediately follows after mitosis, and a G₂-period (6–7 h duration); no G₁-period takes place in *Physarum* macroplasmodia.

The starting point of our investigations was that Chahal, Matthews & Bradbury (1980) described a distinct fluctuation in histone H4 acetate content during the synchronous nuclear division cycle of *Physarum*. In addition, Waterborg, Chahal, Müller & Matthews (1981) reported on a cell cycle dependence of histone deacetylase activity per unit of DNA. The same authors found, that inhibition of histone deacetylase by sodium-n-butyrate in vitro is only weak (Waterborg et al. 1981). After incubation with 2 mM-butyrate the deacetylase still retains 95% of control activity. On the other hand, Jalouzot & Toublan (1981) described a drastic effect of 5 mM-butyrate on *Physarum*, which caused cessation of growth and a block of mitotic plasmodia in metaphase.

These results led to the assumption that histone deacetylase (if at all) is not the only target of sodium-n-butyrate in *P. polycephalum*, but that there might very well be multiple interactions in the cellular metabolism.

**MATERIALS AND METHODS**

**Materials**

The chemicals and radioactive precursors used and their sources were as follows: n-butyric acid, iso-butyric acid and n-valeric acid from Sigma Chem. Co. (London, U.K.); propionic acid from E. Merck (Darmstadt, F.R.G.); succinate-Na from Boehringer Mannheim GmbH (Mannheim, F.R.G.); Packard Instagel from Packard Instrument Co. (Downers Grove, Ill., U.S.A.); [methyl-³H]thymidine (60 Ci/mmol) and [5,6-¹H]uridine (60 Ci/mmol) from The Radiochemical Centre Ltd (Amersham, U.K.); n-[1-¹⁴C]butyrate-Na (14 mCi/mmol) from New England Nuclear (Boston, Mass., U.S.A.).

**Cultures**

Submersed cultures of microplasmodia of *P. polycephalum* (strain M₃b, Wisi isolate) were maintained with shaking in semi-defined nutrient medium (Daniel & Baldwin, 1964) supplemented with 0.013% haemoglobin instead of haematin. Disk-shaped macroplasmodia were prepared by coalescence of exponentially growing microplasmodia on filter paper supported by stainless steel grids in Petri dishes. The time of mitosis (telophase) was determined in smears of small pieces of plasmodium under phase-contrast (Guttes & Guttes, 1964). All culture techniques were carried out under sterile conditions. The sodium salts of acetic acid, propionic acid, butyric acid and valeric acid at pH 7.0 were added to the growth medium. Shortly before addition of the compounds plasmodia were transferred to fresh medium (17 ml for a macroplasmodium). Plasmodia treated with succinate-Na served as an additional control. Mitosis stages 3 and 4 were determined. The control cycle time was 8–9 h.

**Procedures**

**Labelling with radioactive precursors.** Microplasmodia were labelled in separate cultures with [methyl-³H]thymidine or [5,6-¹H]uridine to measure DNA or RNA synthesis by incubating replicate cultures for 30 min in medium containing 2 μCi/ml culture liquid.

**Preparation of samples for DNA, RNA and protein analyses.** Microplasmodia were harvested and washed with distilled water; 5 ml of 5% (v/v) trichloroacetic acid in 50% (v/v) acetone/water were added to the plasmodial sediment before homogenization with a sonifier (MSE, Ltd.
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Crawley, Sussex; low-power, amplitude 1, twice, each for 5 s at 0 °C). After centrifugation for 15 min at 3000 g in a refrigerated centrifuge the pellet was washed once with 5 ml trichloroacetic acid/acetone/water and once with 5 ml of 5 % acid alone. After centrifugation the pellet was resuspended in 3 ml ethanol and centrifuged for 15 min at 5000 g. This pellet was solubilized in 0.5 ml of 0.25 M-KOH. Samples of 0.1 ml were counted in 5 ml Packard Instagel in a liquid scintillation spectrophotometer. Protein analysis was carried out following the procedure of Lowry et al. (1951).

For determination of DNA, RNA and protein contents, without using radioactive precursors, microplasmodia or entire macroplasmodia at selected stages of the nuclear division cycle were harvested and processed as described above, except that after treatment with ethanol the pellet was resuspended in 0.5 ml of 0.025 M-H2SO4 and hydrolysed for 1 h in a boiling-water bath. After centrifugation for 15 min at 5000 g, samples of the supernatant were used for determination of DNA and RNA contents. Analysis of DNA was performed according to the procedure of Gold & Shochat (1980), using cyclohexane for concentration of the chromogen. RNA was measured as described by Ceriotti (1955). The pellet was dissolved in 0.1 M-NaOH and protein was determined according to Lowry et al. (1951).

Thin-layer chromatography of sodium-n-butyrate in growth medium. 1 mM [1-14C]sodium-n-butyrate (200 μCi/mmol) was added to the growth medium. At different times, starting immediately after application, 40-μl samples were taken from the medium and counted in 5 ml Packard Instagel in a Packard Tricarb 2425 liquid scintillation spectrophotometer. In parallel, samples of 40 μl were removed from the growth medium, mixed with 5 μl 0.25 M-NaOH, spotted on TLC-ready aluminium sheets (cellulose; 20 cm x 20 cm; E. Merck, Darmstadt, F.R.G.) and developed for 3 h with a mixture of 4:2:2:2 (by vol.) acetone/iso-butanol/n-butanol/ammonia (1 %). The thin-layer sheets were dried and cut into 1.5 cm wide strips along the migration path; these strips were cut further into 1 cm pieces and the radioactivity was measured after immersion in 5 ml of scintillation liquid (17.5 g PPO and 0.5 g POPOP in 25 l toluene). The Rf-value for sodium-n-butyrate was 0.6 (no additional radioactive metabolites were found in the growth medium at any time point).

RESULTS

Effect of butyrate and homologous fatty acids on synchronous nuclear division

Application of 1 mM-sodium-n-butyrate caused perturbations of the synchronous mitotic cycle of macroplasmodia of P. polycephalum. When butyrate was administered in S- or early G2-period the subsequent mitosis was strongly delayed, whereas application in mid or late G2-period did not influence the timing of the subsequent mitosis (M3), as shown in Fig. 1 A. A significant shortening of the second cycle (M3-Mt), following application of butyrate, could be observed in those cases in which butyrate had been administered in S- or early G2-period of the preceding cycle (Fig. 1 B). Application in mid or late G2-period caused a delay of the second mitosis after start of treatment (M4).

In order to answer the question whether this effect on the nuclear division cycle was specific for butyrate, we tested several other homologous short-chain fatty acids. Fig. 1 A shows that the delaying effect on the subsequent mitosis (M3) after application of the acids increased with the number of carbon atoms. Propionate, isobutyrate and valerate caused a significant prolongation of the second cycle after application. The striking finding was that only n-butyrate could shorten the second cycle (M3-M4), which indicates a specific mechanism of action (Fig. 1 B). The retarding effect on mitosis of all tested compounds is strongly dose-dependent (Table 1).
Fig. 1. Effect of several homologous fatty acids on mitosis 3 (A) and mitosis 4 (B) of macroplasmodia of *P. polycephalum*. At the indicated times (arrows) 1 mM of the sodium salts of acetate (○), propionate (■), n-butyrate (□), isobutyrate (▼) and valerate (▲) were added to the growth medium. Mitosis was determined under phase-contrast microscopy.

Table 1. Effect of various short-chain fatty acids on the nuclear division cycle and synthesis of DNA, RNA and protein in *Physarum polycephalum*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Cycle time (M_4-M_3) (h)</th>
<th>(% \text{DNA} M_3+4 h)</th>
<th>(% \text{DNA} M_4 h)</th>
<th>(% \text{RNA} M_3+4 h)</th>
<th>(% \text{RNA} M_4 h)</th>
<th>(% \text{Protein} M_3+4 h)</th>
<th>(% \text{Protein} M_4 h)</th>
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<tr>
<td>None</td>
<td>—</td>
<td>8.8</td>
<td>175</td>
<td>199</td>
<td>148</td>
<td>190</td>
<td>132</td>
<td>194</td>
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<td>8.9</td>
<td>177</td>
<td>198</td>
<td>149</td>
<td>194</td>
<td>134</td>
<td>195</td>
</tr>
<tr>
<td>n-propionate</td>
<td>1</td>
<td>11.0</td>
<td>177</td>
<td>197</td>
<td>133</td>
<td>178</td>
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<td>218</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.8</td>
<td>161</td>
<td>194</td>
<td>117</td>
<td>195</td>
<td>113</td>
<td>239</td>
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<tr>
<td>n-butyrate</td>
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<td>13.7</td>
<td>161</td>
<td>193</td>
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<td>219</td>
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<tr>
<td></td>
<td>2</td>
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<td>191</td>
<td>113</td>
<td>256</td>
<td>109</td>
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<tr>
<td>Isobutyrate</td>
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<td>196</td>
<td>118</td>
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<tr>
<td>n-valerate</td>
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<td>178</td>
<td>116</td>
<td>224</td>
<td>121</td>
<td>237</td>
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<tr>
<td>Succinate</td>
<td>2</td>
<td>9.0</td>
<td>+</td>
<td>200</td>
<td>+</td>
<td>194</td>
<td>+</td>
<td>200</td>
</tr>
</tbody>
</table>

The DNA, RNA and protein contents at mitosis 2 were taken as 100%. \(M\), mitosis; +, not determined; compounds were added at the time of mitosis 2.
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Effect of butyrate on the synthesis of DNA, RNA and protein in exponentially growing microplasmodia

To characterize further the effect of short-chain fatty acids on Physarum metabolism we studied the effect on the synthesis of DNA, RNA and protein in microplasmodia. Fig. 2A–C shows that the synthesis of DNA, RNA and protein was blocked after addition of n-butyrate. Approximately 12–18 h later recovery was observed and 30 h after the start of treatment the levels of DNA, RNA and protein content reached the control value. These data (Fig. 2A–C) were obtained by determination of absolute contents of DNA, RNA and protein.

Fig. 2. Effect of 1 mM-sodium-n-butyrate on synthesis of DNA, RNA and protein in asynchronous microplasmodia of P. polycephalum. A–C, n-butyrate was added to microplasmodia in submersed culture (4 ml of microplasmodial sediment in 50 ml of nutrient medium) at 0 h. Samples of 5 ml were harvested during the course of the experiment and analysed for total DNA, RNA and protein contents as described in Materials and Methods; ■, control; ○–○, 1 mM-n-butyrate. D. Same experimental protocol as for A–C, except that samples of 2 ml were labelled for 0.5 h with [3H]thymidine or [3H]uridine to measure DNA or RNA synthesis as described in Materials and Methods. Rates of DNA (○–○) and RNA synthesis (■–■) per mg protein are expressed as a percentage of the control.

To get an impression as to how fast the response to butyrate is, the same experiment was done by pulse-labelling of DNA and RNA with radioactive precursors. Fig. 2D shows that immediately after addition of 1 mM-n-butyrate a rapid decline of RNA synthesis to about 10% of the control level took place. Inhibition of DNA synthesis occurred more slowly and reached a maximum (5% of the control) several hours after start of the experiment. Both RNA and DNA syntheses recovered and...
reached control values approximately 30 h after application. Recovery of RNA synthesis was observed earlier than that of DNA synthesis.

Effect of butyrate and homologous fatty acids on the synthesis of DNA, RNA and protein during synchronous nuclear division

After we had measured this drastic effect in asynchronous microplasmodia, we followed the synthesis of DNA, RNA and protein throughout the synchronous mitotic cycle of macroplasmodia in the presence of acetate, propionate, n-butyrate, iso-butyrate, n-valerate and succinate (as additional control) added at the time of mitosis

Fig. 3. Synthesis of DNA, RNA and protein in synchronous macroplasmodia growing in the presence of 1 mM n-butyrate or valerate. The compounds were added to the growth medium at the time of mitosis 2 (M2). At certain times entire plasmodia were harvested for determination of total DNA, RNA and protein contents. The time of mitosis 3 is indicated (arrowheads); ■—■, control; ○—○, n-butyrate; ▲—▲, n-valerate. The DNA, RNA and protein contents at mitosis 2 were taken as 100%.

2 (Table 1). Again we found inhibition of the synthesis of DNA, RNA and protein after application of the drugs. The extent of inhibition was dependent on the applied concentration. Fig. 3 and Table 1 show that the initial inhibitory effect was overcome by the plasmodium during progression in the mitotic cycle. It should be noted that at the time of mitosis 3 the RNA and protein contents of treated plasmodia were considerably higher as compared to controls (except for acetate), whereas DNA reached the control level. Again we observed a dose dependence of elevated RNA and protein levels at mitosis 3 (Table 1).
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To gain some information on the effective lifetime of sodium-n-butyrate in the growth medium we determined the content of butyrate at various times during the mitotic cycle by thin-layer chromatography, as described in Materials and Methods. The concentration of n-butyrate at the time of application at mitosis 2 was 1 mM; within 4 h it decreased slightly to 0.94 mM, 10 h after mitosis 2 it was 0.82 mM and 14 h after application (shortly after mitosis 3) it was 0.73 mM.

DISCUSSION

Recent results on the effects of butyrate in relation to the cell cycle favour the assumption that cells are blocked specifically in the G1-period (D'Anna, Tobey & Gurley, 1980a; Darzynkiewicz, Traganos, Xue & Melamed, 1981; Xue & Rao, 1981; Wintersberger & Mudrak, 1982). The molecular mechanism of this G1-arrest is unknown.

Our approach to this problem was somewhat different, since we tested the sensitivity against butyrate in a system with natural mitotic synchrony. The lack of a detectable G1-period is particularly interesting in view of the data on G1-arrest. First we tried to correlate our observation of maximum delay of mitosis following butyrate treatment early in the cycle with the cell-cycle pattern of histone H4 acetylation (Chahal, Matthews & Bradbury, 1980). Although the percentage of highly acetylated histone H4 is greatest in mid S-phase, which would correspond to our maximum mitotic delay, the fact that there is no butyrate effect on mitosis following application in late G2, in which acetylation of H4 is considerably high, does not favour a causal relation between H4 acetylation and butyrate-induced prolongation of the cell cycle. This seems even more plausible, since 2 mM-butyrate did not significantly decrease the deacetylase activity in vitro (Waterborg et al. 1981). The finding that butyrate has a maximum delaying effect on mitosis when applied during S-phase is in line with the results cited above, since one can assume that G1 events take place during S-phase in Physarum.

It is interesting that we could not arrest plasmodia in mitosis with butyrate, as was recently reported for higher concentrations (5 mM) of butyrate in Physarum (Jalouzot & Toublan, 1981). In our hands butyrate never effected plasmodia in mitosis and a block in metaphase was not found.

Our interpretation of the delay of mitosis in macroplasmodia induced by butyrate is the temporary inhibition of synthesis of DNA, RNA and protein. On the other hand, the elevated RNA and protein levels at the time of the delayed mitosis could perhaps be related to hyperacetylation of histones, since one can imagine that the deacetylase, although only slightly inhibited in vitro, could be inactivated to a greater extent after a period of some hours. This would be in line with data that implicate hyperacetylation of histones with increased template activity of chromatin (Oberhauser et al. 1978; Dobson & Ingram, 1980).

The butyrate-specific shortening of the second cycle after application remains unclear. Whether the induction of new proteins, like the butyrate-enhanced protein (BEP) found in other systems (D'Anna et al. 1980a, b), or one of the numerous other
effects on nuclear function (Boffa, Gruss & Allfrey, 1981), is responsible for this phenomenon needs further investigation.

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REFERENCES


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