Original Article

Biochemical and morphological effects of sodium butyrate on *Dictyostelium discoideum* development

Luis Boto, Amparo Cano and Angel Pestaña¹

Instituto de Investigaciones Biomédicas, CSIC. Facultad de Medicina, UAM. Arzobispo Morcillo, 4. 28029 Madrid, Spain (address for offprints).

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Abstract

Pretreatment of proliferating *D. discoideum* amoebae with 10 mM butyrate for at least 8 h (one duplicating time) induced a reversible and dose dependent premature expression of several developmental parameters when the cells were starved in the absence of the fatty acid. The aggregative phase of the morphogenetic cycle was reduced in 2 h and the appearance of mature fruiting bodies and spores took place 4 h earlier as a result of butyrate pretreatment. Some developmentally regulated proteins, such as contact-sites A, cell surface lectins and cyclic AMP phosphodiesterase were also expressed 2 h earlier in butyrate pretreated cells than in controls. The level of extracellular cyclic AMP was reduced in butyrate pretreated cells, while other parameters of cyclic AMP metabolism were not affected. Butyrate also caused a partial inhibition of growth and the hyperacetylation of histone H4 in growing amoeba. These results suggest that butyrate acts as an inducer of differentiation in *D. discoideum* and can therefore be used as an experimental tool in order to explore regulatory mechanisms operating in slime mold differentiation.

Abbreviations: MES—2-N-morpholinoethanesulfonate; EDTA—ethylenediaminetetracetate; TCA—trichloroacetate; DTT—dithiothreitol; SDS—sodium dodecylsulfate

Introduction

Several reports have shown that n-butyrate and other lipophilic compounds can switch the phenotype of embryonic and transformed cells to the phenotype of differentiating cells [1]. Millimolar concentrations of sodium butyrate reversibly induce several morphological and biochemical modifications when present in the cell growth medium for at least one cell cycle. In one of the most outstanding examples of butyrate action, Friend erythroleukemia cells [2] were induced to differentiate to globin

producing erythroid cells; an effect which was coordinated with the accumulation of new unique sequences of gene RNA transcripts and the induction of new protein synthesis [3]. Butyrate has also been shown to induce hyperacetylation of histones [1, 4] and the synthesis of polypeptide hormones, glycoproteins or enzymes in several cell lines [reviewed in 1].

On the basis of these observations it was hypothesized that butyrate and other inducers of differentiation could be useful for the study of the regulatory mechanisms operating in the develop-

ment of *Dictyostelium discoideum*; a lower eukaryote in which cell proliferation and differentiation are dissociated. Upon starvation, the amoebae move towards attracting centers by a well known mechanism of cyclic AMP relay [5] and subsequently they organize into discrete aggregates from which terminal differentiation to sporocarps occurs [6]. In this report we show that the presence of 10 mM sodium butyrate in the vegetative growth phase of *Dictyostelium* induced a general shortening in the morphogenetic developmental program, when the amoebae were allowed to differentiate in the absence of the fatty acid. These results are discussed in the light of known regulatory mechanisms operating in *D. discoideum* development.

Material and methods

Growth and developmental conditions

Dictyostelium discoideum, strain Ax-2, was axenically grown in HL-5 medium as described [7]. When indicated, n-butyrate or other short chain fatty acids were added to the medium at a final concentration of 10 mM. Cells were usually grown up to densities of $2-4 \times 10^6$ cells/ml. Starvation was initiated by washing the cells three times in distilled water. For development on solid support, washed cells were resuspended at the indicated densities in MES-PDF buffer containing per liter: 1.5 gr of MES (from Sigma), 1.5 gr KCl, 0.6 gr $\text{MgSO}_4 \cdot 6 \text{H}_2\text{O}$, 0.5 mg Streptomycin, pH 6.5 and plated on Millipore filters (HABPO 4700 or 1300) saturated with MES-PDF buffer and supported on Millipore prefilters AP1004700 wetted in the same buffer. Filters were incubated in a humid chamber at 22 °C. For studies in suspension, cells were resuspended in 17 mM Na^+/K^+ phosphate buffer pH 6.0 at the indicated densities, and incubated at 22 °C in a New Brunswick gyratory bath at 140 rpm. Microphotographs were taken on Plus X-120 film (Kodak) using a Nikon Stereoscopic microscope SM with a $4 \times$ plain objective and lateral lighting. Cells or spores were counted in a hemocytometer after dissociation in 17 mM Na^+/K^+ phosphate containing 10 mM EDTA.

EDTA-resistant contacts

The appearance of contact sites A was estimated in cells developing on filters as described [8], except that the developmental medium was the MES-PDF buffer described above. At the indicated time points, cells were dissociated from the filters by vortexing and pipeting in test tubes containing 2 ml of MES-PDF, supplemented with 10 mM EDTA. The dissociated cells were allowed to reaggregate in a gyrotary bath for 20 min at 22 °C. The proportion of cells remaining as single or doublets after this incubation was taken as a measurement of cell adhesion.

Purification and assay of discoidins

Discoidins were obtained from 1×10^8 cells by sonication in ECT buffer (75 mM Na^+ , 75 mM K^+ , 165 mM Cl^- , 1 mM EDTA, 15 mM Tris pH 7.3) followed by centrifugation at $100000 \times g$ for 60 min. Almost 95% pure discoidins were obtained by affinity chromatography of these supernatants on Sepharose-4B [9]. The hemagglutination activity of discoidins in crude extracts or purified preparations was measured with formalinized rabbit erythrocytes using a two-fold serial dilutions in microtiter V-plates, as described [10]. A unit of hemagglutination activity is defined as the last dilution at which agglutination is achieved.

Synthesis of discoidins

Discoidin synthesis was determined by pulse labelling with (^3H) leucine of cells developing in suspension ($3-4 \times 10^7$ cells/ml). Incubation in the presence of 50 microCi of (H^3) leucine (153 Ci/mmol from Amersham) per 10^8 cells was at 22 °C for 2 h in a gyratory bath (140 rpm). After the labelling period, cells were washed three times in ECT buffer and resuspended at the density of 2×10^8 cells/ml in the same buffer. Radioactivity in discoidins was measured after purification as described before and the rate of leucine incorporation in discoidins was corrected for the leucine content of these lectins [9].

Measurement of cyclic AMP

Total cyclic AMP was measured in cells developing in moist filters following the procedure described by Brenner [11], except that the filtration step was omitted. Cyclic AMP recovery was 40% on average as estimated by the addition of a tracer amount of ^3H cyclic AMP to the filters before TCA extraction. Cyclic AMP was measured by a binding assay [12], using the kit supplied by Amersham, with a sensitivity of 0.5 pmole.

Cyclic AMP phosphodiesterase activity

This activity was assayed following the conversion of (^3H) 3'-5' cyclic AMP to (^3H) 5' AMP by homogenates from cells allowed to differentiate on filters. The reaction mixture contained in 0.2 ml, 0.5 microCi of (^3H) cyclic AMP (25 Ci/mmol from Amersham), 15 nmole of unlabelled cyclic AMP, 1 micromole of MgCl_2 , 8 micromole Tris-HCl pH 8.0 and 20 microliter of the whole cell lysate. After 15 min at 22°C in a shaker incubator, the reaction was stopped by boiling for 1 min followed by freezing in ethanol-dry ice. The reaction products were separated by thin layer chromatography [13]. Units of phosphodiesterase activity are given in nmoles of cyclic AMP hydrolyzed under the assay conditions.

Assay of adenylate cyclase activity

At the indicated time intervals cells developing in solid support were detached from the filters by extensive washing with MES-PDF and were then collected by centrifugation. The cell pellet was resuspended in TAES buffer (25 mM Tris-acetate pH 7.5, 1 mM EDTA, 1 M saccharose) at the final density of $1-2 \times 10^8$ cells/ml and homogenized with 50 strokes in a 1 ml glass-glass homogenizer. The assay conditions were basically as described [14], using 0.02–0.03 ml of homogenate in 0.1 ml of the assay mixture containing 1 mM ATP and 0.7 microCi of $(\alpha\text{-}^{32}\text{P})$ ATP. After 10 min at 25°C, the reaction was stopped by the addition of 0.1 ml

of a mixture containing 1% SDS, 1 mM cyclic AMP, 10 mM EDTA, 50 mM Tris-acetate pH 7.5. After the addition of 0.8 ml of distilled water and 0.2 microCi of (^3H) cyclic AMP as a marker for recovery, the reaction mixture was chromatographed as described by Salomon *et al.* [15] and the (^{32}P) cyclic AMP measured by the Cherenkov radiation [16]. Units of adenylate cyclase activity are given in picomoles of cyclic AMP formed under the standard assay conditions.

Isolation of histones

Histones were isolated from nuclear suspensions following the procedure developed by Mende *et al.* [17] for *Physarum polycephalum*, using 40% guanidine HCl for the extraction and ion exchange chromatography in Bio Rex 70 (from Bio Rad). The fraction eluted with 12% guanidine HCl was found enriched in the four core histones as ascertained by SDS acrylamide electrophoresis.

Other analytical methods

Histones were analyzed in polyacrylamide gel slabs using the SDS system described by Laemmli [24] or the urea-acetic acid-Triton from Bonner *et al.* [25]. The gels were stained with Coomassie blue and dried at 40°C between two acetate sheets. The resulting transparencies were scanned with a Quick-scan densitometer. Binding of cyclic AMP to whole cells was assayed using procedures described [26]. Protein was measured by the Lowry [27] and Bradford methods [28] using bovine serum albumin as standard.

Results

Growth of *D. discoideum* in the presence of *n*-butyrate

Cell proliferation of *D. discoideum*, axenic strain Ax2, was diminished when *n*-butyrate was present in the medium. Concentrations of the fatty acid be-

Table 1. Summary effects of short chain fatty acids on *D. discoideum* growth and development.

Treatment ^a	Doubling time ^b (hours)	Streaming ^c (hours)	Contact sites ^d (%)	Spore yield ^e (%)
Experiment I				
Control	8	9.5	n.d.	0.5
Butyrate 1 mM	8.7	9	n.d.	0.6
Butyrate 2.5 mM	9.1	9	n.d.	0.7
Butyrate 5 mM	9.4	8	n.d.	4
Butyrate 10 mM	13.5	7	n.d.	51.6
Experiment II				
Control	8.7	9–10	22	2
Butyrate 10 mM	13.7	7	72	61
Propionate 10 mM	10	9–10	14	30
Isobutyrate 10 mM	10.5	8–9	24	86

^aAmoebae were grown for three days in the presence of fatty acids as indicated. Cells were harvested at densities of $2-4 \times 10^6/\text{ml}$, washed and allowed to develop on Millipore filters as indicated in Material and methods; ^bAverage of three observations; ^cHours after the onset of starvation in which dendritic aggregation streams are seen (two experimental observations); ^dEDTA-resistant cell aggregates as a percent of the total cell in suspension (cells were detached from the filters after 7 h of starvation). Values from one representative experiment; ^eSpores counted after 17 h of starvation as percent of the cells initially plated (triplicate counts from one experiment).

tween 5 and 10 mM increased the doubling time in a 60% on average, from 8–9 hours in control to 13–14 hours in treated cells (Table 1). Lower doses of n-butyrate were without effect; other fatty acid analogues such as propionate and isobutyrate were less effective in retarding growth (Table 1).

The inhibition of growth was manifest without any delay after the addition of n-butyrate (Fig. 1A) and was fully reversed two hours after the transfer of the treated amoeba to a fresh medium lacking the agent (Fig. 1B). A similar two hours lag in the recovery of treated cells was observed (Fig. 1C) during the limited growth period which takes place after starvation on solid support of exponentially growing amoeba [23]. In this case, an appreciable

Fig. 1. Reversible effects of butyrate on *D. discoideum* growth. A) Growth retardation of amoeba grown in the presence of 10 mM sodium butyrate. B) Recovery of amoeba growth after butyrate removal. C) Cell number increase after starvation on solid support of control and butyrate pretreated cells. Average of two representative experiments with triplicate cell counts for each time point. Symbols stand for control (●) and butyrate treated cells (▲).

degree of synchrony was observed, suggesting that butyrate might be retarding some specific step along the cell cycle.

Effect of n-butyrate pretreatment on D. discoideum development

One of the most outstanding effects of butyrate and other inducers is their ability to commit immature or transformed cells to terminal differentiation in the absence of the agent and after a period of exposure to it [24]. In order to explore the presumptive effects of n-butyrate on the terminal differentiation of *D. discoideum*, amoeba grown in the presence of the fatty acid were washed free of nutrients and allowed to differentiate on solid support in the absence of butyrate. Under these conditions it was observed a general shortening of the morphogenetic pathway as compared to controls (Fig. 2). Dendritic cell streaming, the first morphological appearance of the chemotactic and aggregative phenomena in *D. discoideum* differentiation, was consistently observed after seven hours of development of butyrate pretreated cells, two hours earlier than in controls (Fig. 2D and A).

After 17 hours of development most of the aggregates from butyrate pretreated amoeba were close to the final morphogenetic stage of mature sporocarps (Fig. 2F), while the controls were still near the 'mexican hat' stage of development (Fig. 2E). Confirming this speed up of the morphogenetic processes, the spore yield after 17 h of development was very high in pretreated cells as compared to controls (Table 1). No differences were observed in the number or size of the fruiting bodies produced by treated cells as compared to controls and the final spore yield was the same in both cases. These observations would indicate that n-butyrate pretreatment induces a shortening of the normal expression of the morphogenetic program in *D. discoideum*, without affecting the final result of the process. A whole picture of the morphogenetic effects of n-butyrate pretreatment is shown in the scheme at the bottom of Fig. 2.

Requirements for optimal n-butyrate effects

The dose-dependence study of butyrate pretreatment on developmental processes showed (Table 1, Fig. 2A to D) an optimum at the concentration of 5–10 mM. Higher concentrations in the growth medium were deleterious for the cell viability and the presence of the fatty acid during starvation resulted in a considerable retardation of developmental processes (not shown). Other fatty acid analogues such as propionic and isobutyric acid were less effective or had distinct effects on *D. discoideum* growth and development (Table 1).

The developmental effects of butyrate were only observed when the pretreated amoeba were collected in exponential growth (data not shown), suggesting the involvement of cell cycle associated phenomena. Besides this requirement for active growth, the effects of butyrate pretreatment were shown to be dependent on the length of the exposure to the agent, with an optimum of 8–10 h, as ascertained by the criteria of spore production after 18 h of fasting (Fig. 3A) and the reduction of the preaggregative interphase (data not shown).

The reversibility of the morphogenetic effects of butyrate pretreatment also showed a requirement for active growth in the absence of the fatty acid for at least 10 h (Fig. 3B). Interestingly, this study also showed that a maximal effect of butyrate pretreatment on terminal differentiation occurred after 4–6 h of growth in the absence of the drug.

Acquisition of aggregation competence

In order to explore the mechanisms underlying the more rapid expression of aggregation competence in butyrate pretreated cells, the appearance of contact sites A was studied. As shown in Fig. 4A, EDTA resistant cell aggregation was fully developed in butyrate pretreated cells after 7 h of fasting on solid support, 2 hr earlier than in the controls.

Besides contact sites A, which have been identified with membrane glycoproteins [25], two carbohydrate binding proteins – discoidin I and II – are developmentally regulated during the aggregative phase [26] and seem to be implicated in the

Fig. 2. Morphogenetic effects of n-butyrate on *Dictyostelium discoideum*. Amoebae were grown in the absence (A, E) or in the presence of n-butyrate at the concentrations of 1 mM (B), 5 mM (C) and 10 mM (D, F). After three days, cells were washed and allowed to develop on filters as indicated in Material and Methods. Photographs were taken 7 h (A, B, C, D) and 17 h (E, F) after the onset of starvation. The scheme at the bottom of the figure represents the integrated picture of several observations after 10 mM butyrate pretreatment.

cell-adhesion processes in *D. discoideum* [27]. As shown in Fig. 4B, the maximum rate of discoidin synthesis in butyrate pretreated cells was attained after 6 h of development, representing a 7.5% of the total protein synthesized at that time. In con-

trols, the maximum rate of discoidin synthesis took place two hours later and represented a 6.5% of the total protein synthesized in that period. This timing is in good agreement with previous reports showing a maximum label of discoidins between 7–9 h af-

Fig. 3. Minimal time required for n-butyrate action and reversion on terminal differentiation. A) Amoeba were grown in the presence of 10 mM butyrate for the indicated time periods. The washed cells were then allowed to differentiate on solid support. B) Amoeba grown in the presence of 10 mM butyrate for 36 h were washed and resuspended in growing medium lacking the fatty acid. At the indicated time periods, cells were collected, washed and allowed to differentiate in solid support. Spore yield after 18 h of development was recalculated as percent of the number of cells initially plated. Average of at least 5 experimental points $\pm$ standard deviation. * $p \leq 0.05$, ** $p \leq 0.005$ vs time 0 (after 36 h of continuous growth in the presence of 10 mM butyrate).

ter the onset of development of NC-4 cells, amounting to near 6% of the newly synthesized protein in this period [26].

Fig. 4c shows the time course of development of lectin activity as ascertained by hemagglutination assays performed with whole cell homogenates. Those from amoeba pretreated with 10 mM butyrate had a peak of hemagglutination 8 h after the onset of development, two hours earlier than in controls. Similar results were obtained in unpublished experiments in which the temporal expression of discoidin I in cells differentiating on solid support was studied by immunoblotting techniques [27].

Cyclic AMP metabolism in butyrate pretreated cells

The central role of cAMP in *D. discoideum*

Fig. 4. Influence of butyrate pretreatment on the development of the aggregative competence. A) EDTA-resistant aggregation was measured as described in Material and Methods in cells allowed to develop on filters. The results are expressed as the reciprocal of the fraction of cells remaining as single or double after EDTA treatment (0 correspond to the basal value of 0.15–0.20 and 1 correspond to the maximal aggregation of 0.80–0.90). B) Synthesis of discoidins and C) hemagglutination activity in cells developing in suspension after butyrate pretreatment. Beginning at time 0, aliquots were pulse labelled with ^3H leucine at 2 hour intervals. Discoidins were purified and assayed for leucine incorporation as described in Material and Methods. Hemagglutination activity was assayed in crude extracts as described in methods. Symbols stand for cells grown in the absence (●) or in the presence (▲) of 10 mM butyrate for 3 days. In A it is shown the result of one typical experiment out of the six carried out. In B and C it is shown the result of two experiments.

chemotaxis led to a study of cyclic nucleotide levels and related enzyme activities in cells allowed to differentiate on solid support. As shown in Fig. 5A, total cyclic AMP (cellular and extracellular) accumulation in butyrate pretreated cells was

Fig. 5. Cyclic AMP metabolism during differentiation on solid support. Control (●) and butyrate pretreated (▲) amoebae were allowed to develop on filters. Total cyclic AMP (A), adenylate cyclase (B), and cell bound cyclic AMP phosphodiesterase (C) were assayed at the indicated time intervals, as described in Material and Methods. Results in A are average of five independent experiments $\pm$ standard deviation; $**p \leq 0.005$ vs butyrate pretreated after 7 and 8 h of fasting. In B it is shown the average of three experiments. In C it is shown the results of two experiments.

lower and peaked earlier than in controls. Butyrate pretreatment also resulted in a precocious accumulation of cell-bound phosphodiesterase but was without effect on the developmental pattern of adenylate cyclase (Fig. 5B and C). Similar results were obtained in unpublished studies carried out with cells allowed to differentiate in suspension. These studies also showed that the developmental pattern of extracellular phosphodiesterase and cyclic AMP binding were unaffected by n-butyrate pretreatment (data not shown).

Histone acetylation in butyrate treated amoeba

Histone acetylation through inhibition of acetyl group turnover is one of the best known effects of butyrate on eukaryotic cells [1]. In order to study this potential mechanism of butyrate action, a preliminary experiment was designed to ascertain the occurrence of histone acetylation in *D. discoideum*. Isolated nuclei were incubated in the presence of ^3H acetate, and the histones were extracted, analyzed in SDS polyacrylamide electrophoresis. The autoradiographic analysis of the dried gel (data not shown) revealed the presence of acetyl groups in a protein band with a mobility close to the marker H4 histone from calf thymus.

Hyperacetylation of histones in response to butyrate treatment was demonstrated through the electrophoretic analysis of histone subfractions in polyacrylamide slab gels containing urea-acetic acid-Triton [19]. As shown in Fig. 6 the microheterogeneity of histone H4 subfractions was shifted from the high mobility (low acetylation) forms in controls to the slower mobility (high acetylation) forms in butyrate and isobutyrate treated cells. No differences in the degree of acetylation were observed between control cells whether stationary or exponentially growing.

Discussion

The presence of n-butyrate during the vegetative phase of *D. discoideum* has been shown to cause a dose dependent and reversible inhibition of the growth of the amoeba as well as a premature expression of several developmentally regulated parameters upon starvation in a medium lacking the fatty acid. The developmental effects could not be attributed to the persistence of butyrate traces during differentiation since the presence of n-butyrate during development, at concentrations as low as 0.5–1 mM, resulted in a marked inhibition of aggregation and morphogenesis (unpublished). The growth and developmental effects of butyrate in *D. discoideum* are qualitatively similar to those described for HeLa and Friend erythroleukemic

Fig. 6. Sodium butyrate induced microheterogeneity of H4 histones in *Dictyostelium discoideum*. Histone were isolated from *D. Discoideum* amoeba grown under the indicated conditions and electrophoresed in the presence of urea-acetic acid-Triton as indicated in Material and Methods. The figure shows a scan of stained gels with indication of the presumptive degree of acetylation from 0 to 4 residues.

cells [28, 2]. To our knowledge, this is the first report in which n-butyrate was shown to act as an 'inducer of differentiation' in non-mammalian cells.

The precocious expression of EDTA-resistant contact sites in butyrate pretreated cells is in good temporal correlation with the effect of the drug on cell streaming. The synthesis and accumulation of discoidins was also shown to correlate with the morphological developmental effects of butyrate and therefore reinforce the involvement of these lectins in the aggregative phase of *D. discoideum* development. Since discoidin I synthesis is mainly regulated by the accumulation of newly synthesized mRNA transcripts [29], it is not unreasonable to postulate an effect of butyrate at transcriptional

level in *D. discoideum*. This may also account for the premature accumulation of other developmentally regulated proteins such as the cell-bound phosphodiesterase or the glycoproteins involved in the functional expression of contact sites A. In other cell systems, butyrate has been suggested to modulate specific mRNA synthesis by mechanisms involving the transient hyperacetylation of a histone H4 subset [30]. The possible involvement of histone acetylation in the effects of butyrate on *D. discoideum* development awaits further investigation.

The study of cyclic AMP metabolism has shown a marked reduction in cyclic nucleotide accumulation during differentiation, as a result of n-butyrate pretreatment. This finding is rather paradoxical in view of the crucial role of cyclic AMP in cell aggregation and differentiation [8, 31], but can be explained within the current conceptual framework of *D. discoideum* development. In fact, as clearly shown by Wier [32] and Nanjundiah and Malchow [33], the time elapsed between starvation and aggregation is significantly shortened by experimental treatments which lowers the cyclic AMP levels surrounding the amoeba or increase the cell-bound phosphodiesterase activity. Any of these circumstances or the synergistic effect of both – such as in n-butyrate pretreated cells – would prevent the saturation of the cyclic AMP receptors and therefore increase the efficiency of the chemotactic response.

The mechanism underlying the cytostatic effects of sodium butyrate are unknown. In unpublished observations we have found a partial inhibition of amino acids uptake and protein synthesis in growing amoeba by sodium butyrate which could be explained by direct interference of the fatty acid with cell membrane fluidity. It is also possible that butyrate might be directly acting through a reduction in intracellular pH [34], although the differential effects of short chain fatty acids on amoeba proliferation (Table 1) argues against a mechanism of action exclusively based on cytoplasmic acidification.

In contrast to the almost instantaneous effects on cell growth (Fig. 1A, B), the developmental effects of butyrate pretreatment required a time inter-

val of 8–12 h for optimal action and full reversion (Fig. 3A, B). These intervals, which are within the range of the amoeba doubling time (Table 1), suggest the involvement of cell cycle related phenomena in butyrate action. In other cell systems sodium butyrate was shown to inhibit the transition from early G_1 to the subsequent phases in the cell cycle [35]. G_1 cells probably correspond to the light amoeba isolated in isopycnic gradients, which were shown to be more sensitive to cyclic AMP and to form aggregates more rapidly than the heavy cells [36], probably G_2 cells. The importance of cell cycle phase on cell fate during development in *D. discoideum* has been recently emphasized [37, 38]. In view of these observations the putative relationships between cell cycle and developmental effects of sodium butyrate deserve further study.

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