Cyclic AMP, A Nonessential Regulator of the Cell Cycle

(lymphoma/mutants/flow microfluorimeter)

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Communicated by Daniel E. Koshland, Jr., December 9, 1974

ABSTRACT Flow-microfluorimetric analysis has been carried out on populations of exponentially growing S49 mouse lymphoma cells treated with dibutyl cyclic AMP. The drug produces a specific concentration-dependent block in the G1 phase of the cell cycle while other phases of the cycle are not perceptibly altered. The cell cycle of a line of mutant cells lacking the cyclic AMP-dependent protein kinase is not affected by the drug. Since these mutant cells have been shown to maintain a normal cell cycle, even in the presence of high levels of cyclic AMP, periodic fluctuations in the levels of the cyclic nucleotide cannot be required for or determine progression through the cell cycle.

There is considerable evidence that adenosine 3':5'-cyclic monophosphate (cAMP) has a regulatory effect on the growth of cells in culture. The levels of the cyclic nucleotide increase as untransformed cells approach confluency (1, 2), although this is contradicted by others (3). Intracellular cAMP concentrations are negatively correlated with growth rate among a variety of fibroblast cell lines (4). Transformed cells have a lower cAMP content than untransformed cells (1, 5). Exogenous cAMP analogs or the induction of endogenous cAMP slows or stops growth of some cells (6–10). Proliferation of contact-inhibited cells, induced by seeding or proteolytic treatment, is prevented by cAMP analogs (7, 11). The cAMP level changes during the cell cycle and is specifically low in mitosis (11, 12).

While these results make it clear that cAMP can cause marked effects on the cell cycle, important questions remain unanswered.

(i) Is cAMP merely a negative regulator of the cycle, or is there a positive effect of cAMP levels that mediates growth control? Burger et al. (11) have proposed, for instance, that a fall in cAMP is the signal that negatively precedes DNA synthesis.

(ii) What is the locus of the growth-inhibitory action of cAMP? This has been variously reported as lying in G1 (7, 13, 14), in G2 (8), or in discrete portions of the cycle (15, 16). These studies have in most cases used cell populations synchronized by techniques that possibly cause unbalanced growth, which might raise questions regarding the use of these materials for examining the effect of a growth regulatory substance.

To answer these questions we have studied growth regulation in cultured S49 mouse lymphoma cells. These cells are derived from a lymphoma cell line, and it is clear that the drug cAMP does not prevent proliferation of these cells. Furthermore, cAMP has been shown to inhibit growth of other cells and is postulated to be involved in the growth regulation of these cells (17, 18). In this study we have used the flow-microfluorimeter (19) and other techniques to examine the effects of cAMP on growth regulation in exponentially growing populations and have compared the growth inhibitory effect on populations of mutant and wild-type cells. No significant differences were observed between the two populations. In the presence of cAMP, cells of both lines were stopped at the G1 phase of the cell cycle; this inhibition is not required for the normal timing of the cycle.

MATERIALS AND METHODS

N\(^{6},O^{2}\)-Dibutyl cyclic 3':5'-AMP monophosphate (Bt\(_{2}\)cAMP) was purchased from Sigma, theophylline from Calbiochem, and [\(^{3}H\)]thymidine from New England Nuclear Corp.

S49 cells (20) were grown in stationary suspension culture in Dulbecco’s medium with 10% heat-inactivated horse serum in a humidified atmosphere containing 10% CO\(_{2}\). Growth experiments were done with cells in 75 cm\(^{2}\) Falcon flasks containing 20–30 ml of medium. To assure asynchronous growth, cells were maintained without addition of fresh medium for at least 24 hr before an experiment was begun. A mutant subline resistant to Bt\(_{2}\)cAMP was obtained by cloning the cells in soft agar containing the drug (18).

Cells were counted in a Coulter Counter model B. Viable cells excluding trypan blue were determined with a hemocytometer. For autoradiography, cells were labeled for 30 min in [\(^{3}H\)]thymidine, 1 \(\mu Ci/ml\) (specific activity, 21 Ci/mole), washed with cold phosphate-buffered saline (pH 7.4), air-dried on slides, coated with Kodak NTB-2 photographic emulsion, developed, stained, and scored by conventional methods. Between 500 and 1000 cells were examined per sample; positive nuclei labeled densely.

To determine the distribution of cells in the cycle, samples containing 2 to 5 \(\times\) 10\(^{6}\) cells were washed once with cold

<table>
<thead>
<tr>
<th>Cells</th>
<th>Bt(_{2})cAMP + theophylline</th>
<th>% Cells in G(_{1})</th>
<th>Doubling time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>–</td>
<td>34*</td>
<td>18.0</td>
</tr>
<tr>
<td>+</td>
<td>88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cA(^{R})</td>
<td>–</td>
<td>30</td>
<td>16.5</td>
</tr>
<tr>
<td>+</td>
<td>29</td>
<td>16.5</td>
<td></td>
</tr>
</tbody>
</table>

* Measured 24 hr after drug addition.
phosphate-buffered saline and fixed in phosphate-buffered saline containing 10% Formalin. After DNA was stained with acriflavin (21), the cells were analysed in the Lawrence Livermore Laboratory flow-microfluorimeter; the apparatus is described in detail elsewhere (22). Briefly, stained cells are hydrodynamically focused in the flow-microfluorimeter, so that they travel in single file, at rates up to 10^4/sec, through the intense beam of exciting light from an argon ion laser. The acriflavin in each cell is thus stimulated to fluoresce; this light is detected by a photomultiplier and converted into an electrical pulse, which is amplified and subsequently stored in a multichannel analyzer. After analysis of a large number (about 10^5) of cells, the contents of the analyzer represents the DNA distribution of the population.

The distribution of cells among G1, S, and G2 + M can be calculated from the DNA histograms (23). The duration of each phase of the cycle can then be measured from the doubling time of an exponentially growing population whose growth fraction is unity, assuming that the doubling time equals the generation time and that the population age distribution decreases exponentially with age (24). Computer modeling of exponential steady-state cell growth and kinetic analysis of perturbed populations were done as described by Gray (25).

**RESULTS**

Cell Growth Inhibited by Bt2cAMP. Cloned S49 cells in exponential growth were treated with 0.2 mM theophylline and the indicated concentrations of Bt2cAMP, and the cell density was measured as a function of time (Fig. 1). The untreated
control cells grew with a doubling time of 17-18 hr. After addition of theophylline and 0.1 mM Bt2cAMP, the cell density continued to increase at the same rate as the control for approximately one generation time and then remained constant. The growth arrest occurred when the cell number was double that present at the time of drug addition, regardless of the initial cell density, so long as the culture began in exponential, i.e., asynchronous, growth. After 24 hr of treatment the cells were fully viable, as measured by trypan blue exclusion and the resumption of growth upon resuspension in fresh growth medium. After 48 hr, the viability was about 20-50% and declined rapidly thereafter. Lower concentrations of Bt2cAMP reduced the growth rate after a lag time of about one cell generation (Fig. 1). These results suggest that Bt2cAMP inhibits growth in a specific phase of the cell cycle.

To study this question, we analyzed treated and control cells with the flow-microfluorimeter to determine the distribution of the cell population among the G1, S, and G2 + M phases of the cycle. Application of this method revealed that the phase durations were 2.1, 12.0, and 3.0 hr for Tg1, Ts, and TG2+ M, respectively, in wild-type S49 cells. Table 1 shows the doubling time and fraction of cells in G1 for both the wild-type and a mutant subline unresponsive to cAMP due to a defect in cAMP-dependent protein kinase (17, 18). After treatment with Bt2cAMP and theophylline for 24 hr, the mutant cells were unaffected in doubling time or cell cycle distribution. The wild-type cells, however, had ceased to grow and the fraction of cells in G1 had increased from 34 to 88%. It should be noted that the kinetic parameters of the mutant cells differed slightly from those of the wild-type cells.

G1 Prolonged by Bt2cAMP. To follow the kinetics of this perturbation in cell cycle distribution, we treated exponentially growing cells as in Fig. 1 with 0.1 mM Bt2cAMP and 0.2 mM theophylline and, periodically, samples were collected for flow-microfluorimeter analysis (Fig. 2). After 2 hr of treatment, the cell cycle distribution seen in the flow-microfluorimeter (Fig. 2A) did not differ significantly from that in the control untreated population (not shown). By 4 hr a relative decrease in the early-S population was apparent, which became more marked at 6 hr and extended progressively to the late S population at 8 and 10 hr. At 12 hr, the number of G2 cells began to fall; by 18 hr more than 90% of the cells were in G1.

It is apparent from these data that Bt2cAMP imposes a G1 block, and this impression is confirmed by computer-generated DNA histograms (Fig. 2B). These were derived by modeling (25) an exponentially growing population using cell cycle parameters obtained by flow-microfluorimeter analysis of untreated cells. The model was perturbed only by imposing a block in early G1 and following the change, with respect to time, of the cell cycle distribution of the model population. Computer models assuming a block in late G1 did not reproduce the experimental data. Comparison of Fig. 2A and B shows that the match is good between experimental and computer-generated data.

Other Cell Cycle Parameters Not Affected by Bt2cAMP. Experiments were done to exclude the possibility that Bt2cAMP has another site of action in the cell cycle, and to show that cells that have not yet reached the block, or have escaped from it, cycle normally. The modeling results in Fig. 2 support this hypothesis since they were generated assuming only a block in G1. To test this idea further, cells were pulse-labeled with [3H]thymidine at intervals after treatment with Bt2cAMP and theophylline, and autoradiograms prepared to determine the fraction of labeled cells (Fig. 3). These data were compared to the fraction of cells in S generated by the cell cycle model, again assuming only a block in early G1. The good agreement between experimental and computer-generated data provides an independent confirmation of the conclusions from the flow-microfluorimeter results. Hence, S is of normal duration for at least one generation after drug addition.

Dose-dependent Partial Block Imposed by Bt2cAMP. As shown in Fig. 2, there was a residual S and G1 + M population after 18 hr of treatment with 0.1 mM Bt2cAMP and 0.2

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**Fig. 3.** Fraction of cells labeled with [3H]thymidine after treatment with theophylline, 0.2 mM, and Bt2cAMP, 0.5 mM. Points represent experimental data; the line represents data generated by a computer model that assumes an early G1 block (see text).

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**Fig. 4.** Effect of colcemid on the cell cycle distribution of Bt2cAMP-arrested cells. Samples were analyzed by flow-microfluorimeter after the following treatments: A, none; B, colcemid, 1 μg/ml for 10 hr; C, Bt2cAMP, 0.1 mM, and theophylline, 0.2 mM, for 20 hr; D, Bt2cAMP and theophylline for 20 hr with addition of colcemid for a further 10 hr.

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mM theophylline; these cells are present even after 48 hr of treatment with 0.5 mM Bt2cAMP and 0.2 mM theophylline. This minor cell population not blocked in G1 could represent a distinct subclass not responsive to cAMP. This is unlikely because the cells had been recently cloned and only about one in 10⁶ cells are mutants resistant to the growth-inhibitory and cytolytic effects of Bt2cAMP (18).

Other explanations for the presence of cells not blocked in G1 include the possibility that they are dead, that they are cells subject to a secondary block in other parts of the cycle, or that cells infrequently but regularly escape the G1 block. All but the last of these would be excluded if it could be shown that the residual S and G2 + M populations are cycling normally after prolonged drug exposure.

To test this, cells were grown with Bt2cAMP and theophylline for 20 hr and colcemid was then added to the culture for a further 10 hr, to accumulate cells in G2-mitosis (Fig. 4). A control culture, in exponential growth, was treated with colcemid alone for 10 hr. Flow-microfluorimeter samples were prepared before and after colcemid addition. As expected, the control culture after treatment with colcemid contained only a small late S population and a large population of cells with G2 DNA content, representing cells arrested in mitosis. If the Bt2cAMP-treated culture contained cycling S and G2 cells, an increase should be seen in the G2 + M peak as cells transit into mitosis and are arrested there. This was observed. Indeed, there was an approximately 3-fold increase in the area of the G2 + M peak, as expected for a population with G2 + M duration of 3.0 hr accumulating mitotic cells for 10 hr. The DNA distributions of control cells treated with Bt2cAMP and theophylline did not change between 20 and 30 hr. This confirms as well that the duration of G2 + M is unaffected by the block.

Therefore, since even high Bt2cAMP concentrations induce a "leaky" block, experiments were done to investigate the relationship between drug dose and the effectiveness of G1 arrest. Cells were exposed to different concentrations of Bt2cAMP in the presence of 0.2 mM theophylline for 36 hr, so that a nearly steady-state cell cycle distribution was achieved. The fraction of the cell population in G1 was determined by flow-microfluorimeter as an index of the effectiveness of the block (Fig. 5). The fraction of cells in G1 was linearly related to the exogenous Bt2cAMP concentration in the range 10⁻⁸–10⁻⁴ M. The effect of added 10⁻⁴ M Bt2cAMP was small but significant compared with the control treated with theophylline alone.

**DISCUSSION**

Previous work has shown that cAMP induced endogenously by hormones or added exogenously as the dibutyril analog inhibits the growth of S49 cells and eventually kills them (17, 18). The present experiments show that growth inhibition is caused by prolonging the mean duration of G1 without significantly affecting the length of S, G2, or M. Although high concentrations of Bt2cAMP prolong G1 to a degree simulating complete growth arrest, the cells that emerge from G1 traverse the rest of the cycle with kinetics similar to those of exponentially growing cells. Thus, cells that escape the block are not members of a distinct subpopulation.

Is the effect demonstrated here due directly to cAMP? The G1 inhibitory effect of Bt2cAMP can be reproduced by raising the endogenous cAMP level in S49 with cholera toxin, a stim-

![Fig. 5. Fraction of cells in G1 36 hr after treatment with varying Bt2cAMP concentrations. All cultures contained 0.2 mM theophylline. The fraction of cells in G1 was determined by flow-microfluorimeter analysis as described in the text.](image-url)
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