Arrest of Cultured Rat Liver Cells in G2 Phase by the Treatment with Dibutyryl cAMP

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When rat liver cells which had been grown in a protein- and lipid-free synthetic medium were incubated in the presence of dibutyryl adenosine 3':5'-cyclic monophosphate (But_2cAMP) and theophylline, labeling index of cells with [^3H]-thymidine was decreased. Percentage of cells which had twice the amount of DNA per nucleus compared to the basic value increased during the cultivation of cells in the presence of the drugs. Mitotic index increased immediately after the removal of the drugs. From these results, it was concluded that But_2cAMP arrested the cell cycle mainly at G2 phase.

INTRODUCTION

Adenosine 3':5'-cyclic monophosphate (cAMP) has been considered as one of the factors which control the growth of cells (1-5). When cAMP or its dibutyryl derivative (But_2cAMP) was added into the cultures of various kinds of growing cells, cell growth was readily inhibited. Intracellular concentration of cAMP increased when cell sheets became confluent, and decreased when the cells were shifted from the resting to the growing state (6, 7).

Sheppard reported that cAMP restored the contact-inhibited growth of transformed cells cultured in vitro (2). Contact inhibited cells were arrested at the G1 phase of cell cycle (8, 9). The results, however, of the effects of cAMP on cell cycle obtained by several authors were conflicting with each other; Teel & Hall (10) reported that cAMP

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arrested the cells at G1 phase, whereas Remington & Klevecz (11) showed that it blocked cell cycle at G2 phase.

In the present communication, we are showing that But$_2$cAMP blocks the cell cycle at G2 phase.

MATERIALS AND METHODS

Cell Strain and Medium: A cell strain used in this experiment, JTC-25·P5 (12), was originated from rat liver parenchymal cells which had been transformed by "Nagisa culture" (13). The cells had been adapted to grow in a protein- and lipid-free synthetic medium (12), and serially propagated in Eagle's MEM (14, a commercial product of Nissui Seiyaku Co., Tokyo) in the absence of proteins. Cells were grown in monolayer.

Measurement of Mitotic Index and Labeling Index: Cells were inoculated into short test tubes in a cell density of 3×10^5 cells/ml/tube, and were incubated in stationary culture at 37°C being kept slanted at an angle of 5°. But$_2$cAMP and theophylline were added to each tube in concentrations of 0.25 mM and 1.0 mM, respectively.

For the mitotic index analysis, cells were incubated for 4 days and transferred into fresh medium without the drugs. Cells were collected at certain intervals and were dislodged by a rubber policeman in 2.1% citric acid solution with 0.05% crystal violet. Number of cell nuclei in mitosis and in interphase were counted with hemocytometer. About 500 to 1,000 nuclei were counted at each point.

For the labeling index analysis, cells which had been grown on glass coverslips (cell density = 3×10^5/ml/tube) were incubated with ^{3}H-thymidine (0.5 μCi/ml, 5-methyl-[^{3}H], 5.0 Ci/mmole) for 1 hr at each point after the addition of drugs. Coverslips were taken out, washed three times with buffered saline, fixed with a cold mixture of methanol and acetic acid (1 : 1 by volume), washed with cold 5% perchloric acid, washed with water and finally covered with emulsion (Sakura Co., NR-M2). Exposure time was 7 days.

Impulse Cytophotometric Analysis of Relative Content of DNA per Nucleus: Cells were incubated for 4 days in the medium containing 1 mM theophylline or 1 mM theophylline plus 0.25 mM But$_2$cAMP, and they were washed and suspended in saline. The resultant cell suspension was fixed with 95% ethanol and centrifuged (2,000 r.p.m. for 10 min). The precipitate was treated with 0.1% Triton X-100 (Daiichi Pure Chemicals, Tokyo) at room temperature for 10 min, and centrifuged. The precipitate was suspended in 0.1 M Tris, pH 7.4, containing 10 μg/ml of ethydium bromide for fluorescent staining of DNA. Distribution of DNA content per nucleus in the population was analyzed with a pulse cytophotometer (Pywe ICP 11, West Germany).

RESULTS AND DISCUSSION

Changes in labeling index of JTC-25·P5 cells after the addition of drugs are shown in Fig. 1. Labeling index gradually decreased when the cells were incubated with theophylline or theophylline plus But$_2$cAMP. The rate of decrease was larger in the latter group than in the former group.

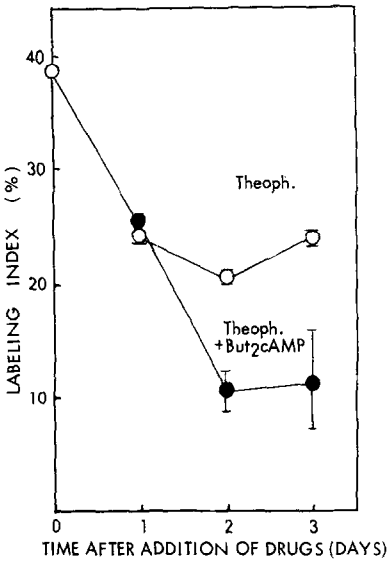

Fig. 1. Changes in labeling index after the addition of drugs. Cells of JTC-25·P5 were grown on coverslips. 1 mM Theophylline (o——o) or 1 mM theophylline plus But2cAMP (●——●) was added on 0 day. Labeling index was obtained by labeling the cells with [^{3}H]-thymidine for 1 hr at each point.

Fig. 2 shows the distribution of DNA content per nucleus as revealed by a pulse cytophotometer. Percentage of cells containing twice the amount of basic DNA increased when the cells were treated with But2cAMP. The similar results were reported by Smets (15). From the pattern of Fig. 2, it was calculated that the fraction of cells which contained twice the amount of DNA (possibly in G2 phase) was 39.8% of the total population when treated with But2cAMP and theophylline, whereas it was 19% when treated with theophylline alone.

Cells which had been incubated in the medium containing 1 mM theophylline in the absence or presence of But2cAMP for 4 days were washed and incubated in a fresh, drug-free medium. Mitotic index was determined at certain intervals. The results shown in Fig. 3 indicate that mitotic index increased immediately after the removal of the drugs but decreased after the 2nd hr.

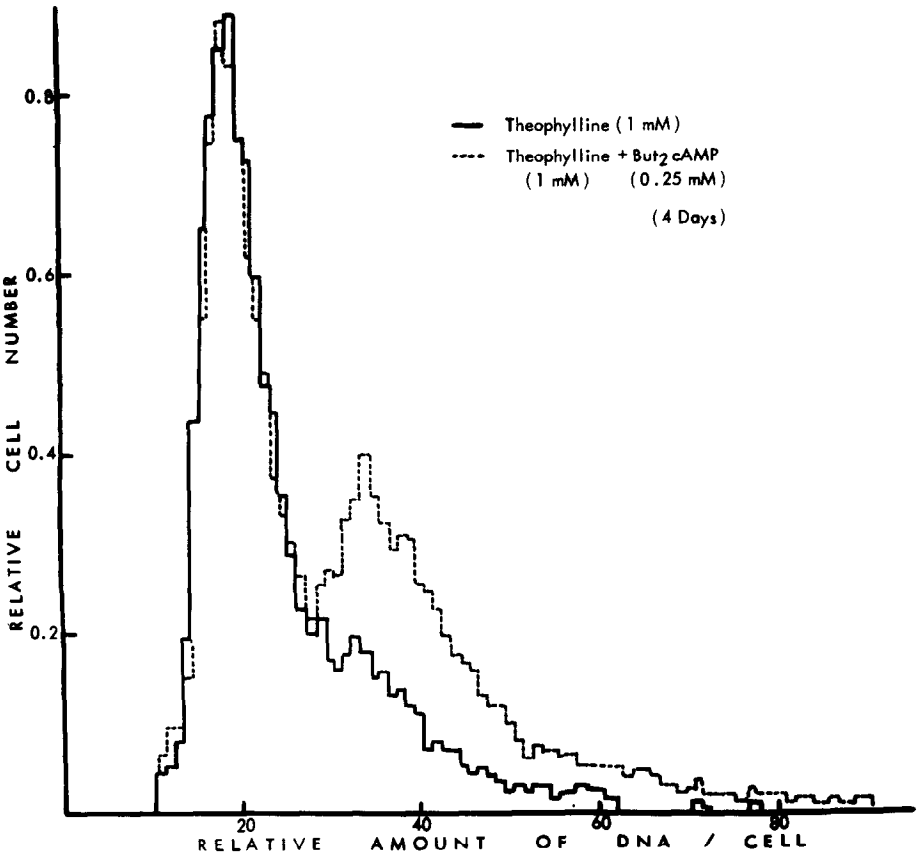

Fig. 2. Pulse cytophotometric analysis on relative amount of DNA content of JTC-25·P5 cells. Cells were treated with 1 mM theophylline (——) or with 1 mM theophylline plus 0.25 mM But$_2$cAMP (- - - -). Abscissa: Channel number of a cytophotometer which is proportional to relative amount of DNA per cell; ordinate: relative number of cells (arbitrary units). 15,000 cells were measured in each experiment.

From the results of Figs. 2 and 3, it was concluded that the number of cells in G2 phase increased among the population of But$_2$cAMP-treated cells.

The effects of cAMP on cell cycle have been investigated by the use of various kinds of cell strains. Phenomena so far as known were (i) inhibition of mitosis (16), (ii) arrest of cell cycle in G1 phase (10), and (iii) inhibition of DNA synthesis (16, 17). In our work, with cultured rat liver cells, it was shown that But$_2$cAMP and theophylline

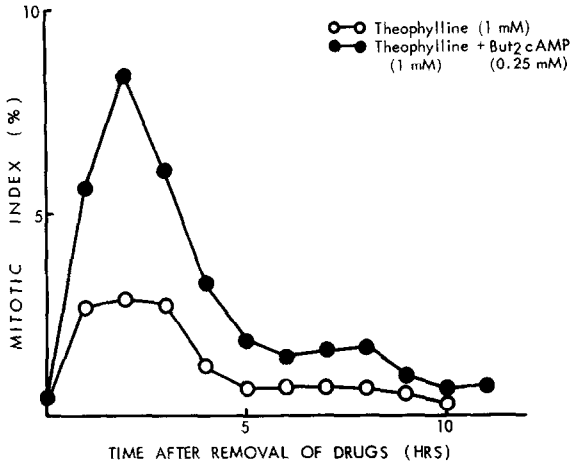

Fig. 3. Changes in mitotic index after removal of the drugs. Cells were treated with 1 mM theophylline or with 1 mM theophylline plus But2cAMP for 4 days. Cells were washed and transferred into fresh medium. Mitotic index was measured at times.

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arrested the cell cycle in G2 phase. These discrepancies might be due to the differences in techniques and in cell strains employed.

Intracellular level of cAMP changed during cell cycle (18 - 20), and was highest at S and G1 phases. Several growth-inhibiting substances such as bleomycin (21) and aminonucleoside (22) were also known to block the cells at G2 phase. From these observations, the cessation of cell growth caused by the addition of cAMP would presumably be due to a different mechanism from that of so-called "contact inhibition" which was understood by some workers to be the arrest of cell cycle at G1 phase.

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