

INHIBITION OF GROWTH AND DNA SYNTHESIS IN CELL CULTURES BY CYCLIC AMP

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SUMMARY

Cyclic AMP (0.1 to 1 mM) was found to inhibit the growth of human liver cells in monolayer cultures. Significant amounts of degradation products were not detected in the medium indicating that the growth-inhibiting effect was associated with the intact cyclic nucleotide. DNA synthesis in the liver cell cultures, as measured by thymidine incorporation into acid-insoluble material, was markedly inhibited by cyclic AMP. RNA and protein synthesis were not significantly affected. Cyclic AMP induced a considerable increase in the cellular uptake of thymidine and uridine from the medium.

When the liver cells were incubated in medium containing radioactive cyclic AMP, no labelled cyclic AMP could be detected in the acid-soluble cell fraction by chromatographic analysis. It is suggested that cyclic AMP does not enter the liver cells, but that its action on growth and DNA synthesis is somehow mediated through an interaction with the cell surface.

INTRODUCTION

Recent studies indicate that cyclic 3',5'-adenosine monophosphate may play an important role in the regulation of cellular growth. Thus, the cyclic nucleotide affects the proliferation of various types of cells in tissue culture (Bürk, 1968; Ryan & Heidrick, 1968; Heidrick & Ryan, 1970, 1971; Ryan & Durick, 1972) and it has been shown that under a variety of conditions the intracellular level of cyclic AMP is inversely related to the growth rate (Otten, Johnson & Pastan, 1971, 1972; Sheppard, 1972; Burger, Bombik, Breckenridge & Sheppard, 1972; Seifert & Paul, 1972). Moreover, it has been reported that cells transformed by oncogenic viruses have a lower level of cyclic AMP than uninfected cells (Sheppard, 1972; Otten, Bader, Johnson & Pastan, 1972; Burger *et al.* 1972; Seifert & Paul, 1972) and that addition of dibutyryl cyclic AMP may restore controlled growth in transformed cells (Sheppard, 1971).

In attempts to elucidate the mechanism whereby cyclic AMP is able to influence cell proliferation, the effect of the cyclic nucleotide on growth, DNA, RNA and protein synthesis in human liver cell cultures has been investigated.

MATERIALS AND METHODS

Cell culture

All experiments were carried out with Chang human liver cells, grown in monolayer cultures under conditions described previously (Eker, 1965), except that the serum used was heated at 70 °C for 15 min to inactivate phosphodiesterase.

Analytical

Cell counts were carried out with an automatic particle counter. Acid-soluble and -insoluble cell fractions were prepared by extraction with 1 ml of cold 5% trichloroacetic acid (TCA) for 15 min at 0 °C, followed by filtration through a Millipore filter (0.45 µm). The filters were washed 5 times with 5 ml of cold TCA, dried at 80 °C for 1 h and the radioactivity determined as described by Olsnes (1970). The radioactivity in the TCA-soluble fraction was measured by transferring aliquots to liquid scintillation vials containing 10 ml of Instagel (Packard) and counting in a Beckman LS-150 liquid scintillation counter.

The distribution of radioactivity in the acid-soluble fraction of the cells was determined by descending paper chromatography in 1 M ammonium acetate (pH 5) and 95% ethanol (3:7; v/v) at room temperature for 18 h (Schröder & Plagemann, 1971). Appropriate standards were applied to the chromatograms and their positions after chromatography were located by examination of the paper under ultraviolet light. The chromatograms were cut into 1-cm segments which were eluted with 1 ml of distilled water at room temperature for 30 min. The eluates were analysed for radioactivity as described above.

Chemicals

[8-³H]adenosine-3',5'-cyclic monophosphate, ammonium salt (20.7 Ci/mmol), [6-³H]thymidine (2 Ci/mmol), [5-³H]uridine (5 Ci/mmol), and uniformly labelled L-[U-¹⁴C]alanine (10 mCi/mol) were obtained from the Radiochemical Centre, Amersham, England. All the other chemicals used in the present work were purchased from Sigma.

RESULTS AND DISCUSSION

The effect of cyclic AMP on growth of human liver cells in monolayer cultures is shown in Fig. 1. It can be seen that the presence of the cyclic nucleotide in the medium at a concentration of 0.5 mM arrested cell multiplication after 24 h.

The lowest concentration of cyclic AMP found to affect cell growth was 0.1 mM. Cells which had been treated with cyclic AMP for 24 h at a concentration of 1 mM rapidly resumed growth when the cyclic nucleotide was removed from the medium. This finding indicates that the action of cyclic AMP on the liver cells was reversible and that no permanent damage was induced by the treatment. Similar results have previously been obtained with L cells (Ryan & Heidrick, 1968).

In liver cell cultures grown on medium containing non-inactivated serum, exogenous cyclic AMP was rapidly hydrolysed by phosphodiesterase. Therefore, in all the present experiments the serum was heat-treated to inactivate the cyclic AMP phosphodiesterase. Nevertheless, a small breakdown of the cyclic nucleotide might possibly occur. To study whether the observed inhibition of growth could be due to extracellular formation of toxic metabolites of cyclic AMP, the distribution of radioactivity in the medium was analysed after incubating the cells with labelled cyclic AMP (1 mM) for different periods of time. The recovery of label in the medium

during the experimental period was about 98%. After 4 h, 98% of the total radioactivity in the growth medium was recovered as cyclic AMP, and only about 2% (0.02 mM) was identified as adenosine. After 24 and 48 h, 4% (0.04 mM) and 7% (0.07 mM), respectively, were recovered as adenosine. Other degradation products of the cyclic nucleotide could not be detected in the medium. Since it was found in other experiments that a concentration higher than 0.2 mM of adenosine was required to affect growth of the liver cells, it can be concluded that the inhibiting effect of cyclic AMP on cell division is not due to the formation of toxic metabolites in the medium, but must be associated with the cyclic nucleotides as such.

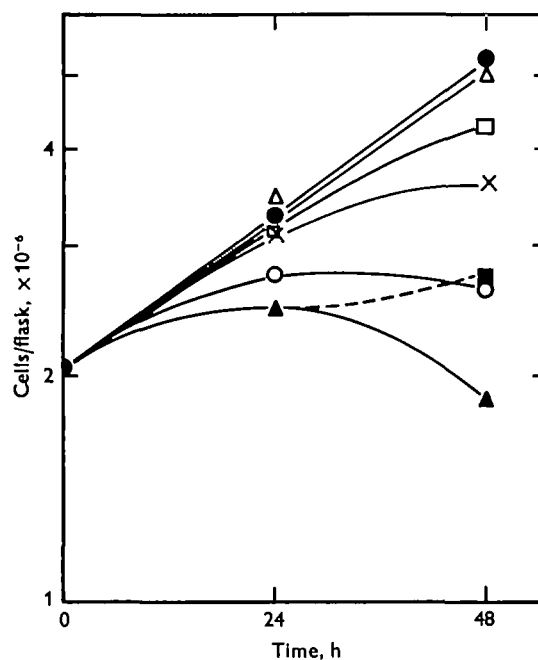


Fig. 1. Effect of cyclic AMP on cell growth. The medium of exponentially growing monolayer cultures was replaced by fresh medium containing different concentrations of cyclic AMP. Cell counts were performed after 24 and 48 h of incubation at 37 °C. The dashed line (■) shows cell growth after removal of cyclic AMP from the medium. ●, Control; △, □, ×, ○, and ▲, with 10^{-5} , 10^{-4} , 3×10^{-4} , 5×10^{-4} and 10^{-3} M cAMP, respectively.

Heidrick & Ryan (1971) and Ryan & Durick (1972) have reported the presence of radioactive cyclic AMP in Strain L cells after prolonged incubation in medium containing ^3H -cyclic AMP. On the other hand, Schröder & Plegemann (1971) were not able to detect any significant uptake of cyclic AMP by various cell lines.

To determine whether the cyclic nucleotide was taken up by the liver cells, labelled cyclic AMP was added to the culture medium and the distribution of radioactivity in the acid-soluble fraction was analysed by paper chromatography after incubation of the cells for various periods of time. The results in Fig. 2 show that after 1 h of

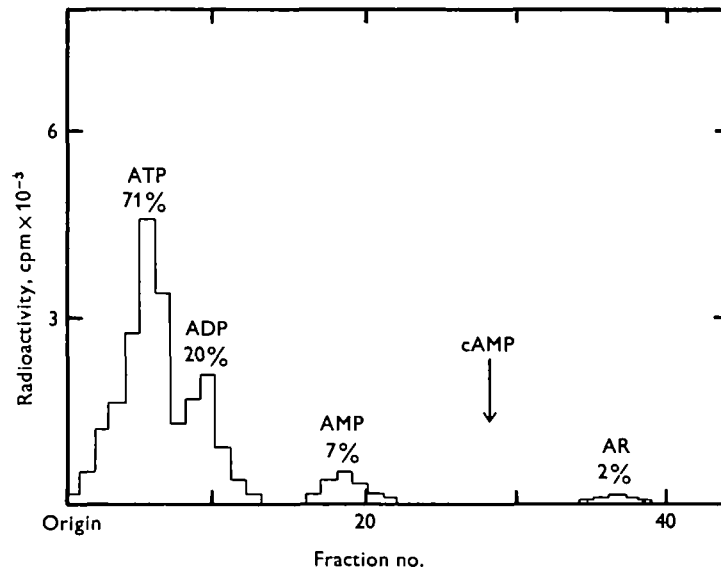


Fig. 2. The distribution of radioactivity in cells after 60 min of incubation in medium containing labelled cyclic AMP. ^3H -cyclic AMP ($10 \mu\text{Ci}$) was added to the medium of exponentially growing cell cultures. After 1 h of incubation at 37°C the distribution of radioactivity in the acid-soluble fraction of the cells was determined as described in Materials and methods. The arrow indicates the position of the reference cyclic AMP.

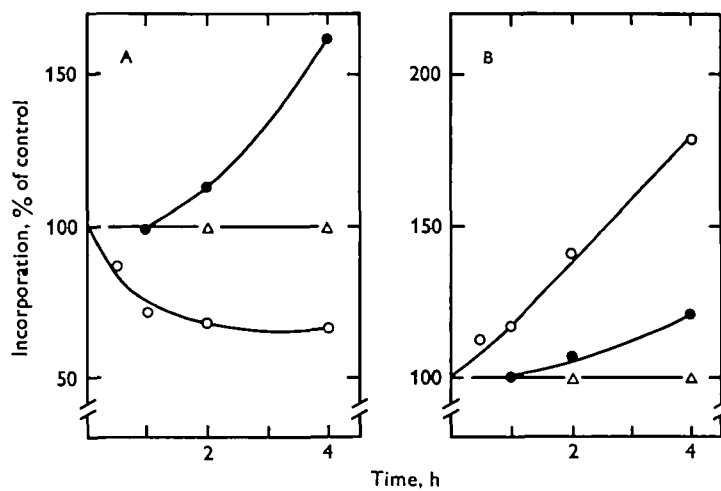


Fig. 3. Effect of cyclic AMP on DNA, RNA and protein synthesis in liver cells. The medium of exponentially growing cultures was replaced by medium containing 1 mM cyclic AMP. After different periods of time the radioactive compounds were added to the medium and the cultures were incubated for 30 min. The radioactivity in the acid-insoluble (A) and acid-soluble (B) fractions of the cells was determined as described in Materials and methods. ●, ^3H uridine; Δ, ^{14}C alanine; ○, ^3H thymidine.

incubation, 71 % of the radioactivity in the acid-soluble cell fraction was identified as ATP, 20 % as ADP, 7 % as AMP and 2 % as adenosine. No trace of radioactive cyclic AMP could be detected. Similar results were obtained when the cells were incubated for 2 and 4 h.

Failure to detect labelled cyclic AMP inside the liver cells might be due to a rapid intracellular breakdown by phosphodiesterase. Therefore, experiments were also carried out with cells which had been preincubated for 60 min in medium containing 10 mM theophylline, an inhibitor of cyclic phosphodiesterase (Robinson, Butcher & Sutherland, 1971). Even then, no labelled cyclic nucleotide could be detected inside the liver cells. This finding indicates that cyclic AMP does not penetrate the membrane of the liver cells. The radioactive nucleotides found in the acid-soluble cell fraction are probably formed intracellularly from labelled adenosine which is present in the medium in small amounts, and which is known to enter cells rapidly.

Table 1. *Effect of cyclic AMP on phosphorylation of thymidine*

Concentration of cyclic AMP, mM	Time, h	Distribution of radioactivity in acid-soluble fraction, % of total			
		TdR	dTMP	dTDP	dTTP
0 (control)	2	1	10	15	74
1	2	1	11	12	76
0 (control)	4	2	8	11	79
1	4	1	10	13	76

The medium of exponentially growing monolayer cultures was replaced by medium containing 1 mM cyclic AMP and incubated at 37 °C. After different periods of time [³H]thymidine (10 μCi) was added and the cultures were incubated for 30 min. The distribution of radioactivity in the acid-soluble cell fraction was determined as described in Materials and methods.

In attempts to elucidate the mechanism of the growth inhibition by cyclic AMP, its effect on DNA, RNA and protein synthesis was studied. From the results in Fig. 3 it appears that the presence of cyclic AMP in the growth medium caused a marked inhibition of the incorporation of labelled thymidine into the acid-insoluble fraction of the liver cells during the first 4 h of incubation. During this period the cyclic nucleotide also induced a considerable increase in the cellular uptake of labelled thymidine from the medium into the acid-soluble pool fraction.

The inhibition by cyclic AMP of thymidine incorporation into DNA of the liver cells might be due to inhibition of the phosphorylation of thymidine to the corresponding triphosphate. This possibility seems to be ruled out by the results in Table 1, which show that after incubation of the cells in medium containing labelled thymidine, about the same amount (75–80 %) of the total radioactivity was recovered as dTTP in the acid-soluble fraction of untreated and cyclic AMP-treated cells.

Fig. 3 also shows that treatment of the liver cells with cyclic AMP leads to an increase in the incorporation of labelled uridine into the acid-insoluble fraction,

suggesting an increase in the rate of RNA synthesis. However, it should be noted that a concomitant rise occurred in the cellular uptake of labelled uridine from the medium into the acid-soluble fraction, which is at least partly responsible for the rise in radioactivity of the acid-insoluble cell fraction.

The uptake of labelled alanine from the medium into the acid-insoluble and acid-soluble fractions of the liver cells was not affected by treatment with cyclic AMP (Fig. 3). This indicates that the protein-synthesizing system of the cells is not influenced by the cyclic nucleotide.

The mechanism by which cyclic AMP inhibits the proliferation of mammalian cells in tissue culture is unknown. The present results indicate that the cyclic nucleotide does not penetrate the membrane of the liver cells, but may somehow elicit its action on cell growth and DNA synthesis at the cell surface. As discussed by Robinson *et al.* (1971) there is some evidence to indicate that the effect of exogenous cyclic AMP may possibly be mediated by a stimulatory effect on the membrane-bound adenylyl cyclase. Whether such a mechanism may account for the present results is being investigated.

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