[CANCER RESEARCH 33, 2104-2108, September 1973]

Inhibition by $N^6, O^{2'}$-Dibutyryl 3',5'-Cyclic Adenosine Monophosphate of Phosphate Transport and Metabolism in $BHK_{21}C_{13}$ and $BHK_{21}Py$ Cells

C. Blat, N. Boix, and L. Harel

Institut de Recherches Scientifiques sur le Cancer, Boite Postale 8, 94800 Villejuif, France

SUMMARY

Theophylline plus dibutyryl 3',5'-cyclic adenosine monophosphate (dibutyryl cAMP) decreases the multiplication rate of both $BHK_{21}C_{13}$ and $BHK_{21}Py$ cells. The level of saturation density of $BHK_{21}C_{13}$ cultures is diminished in the presence of theophylline plus dibutyryl cAMP. On the contrary, dibutyryl cAMP plus theophylline does not prevent $BHK_{21}Py$ cultures from reaching the high saturation density observed in the absence of nucleotide.

Dibutyryl cAMP plus theophylline or dibutyryl cAMP alone inhibit both phosphate transport and metabolism in normal as well as in transformed cells, but the mean phosphate uptake is more inhibited in $BHK_{21}C_{13}$ cells than in $BHK_{21}Py$ cells. The results agree with the hypothesis that 3',5'-cyclic adenosine monophosphate is an intermediate in the contact inhibition of growth.

INTRODUCTION

Heidrick and Ryan (4) and Sheppard (15) have shown that cAMP[1] or dibutyryl cAMP inhibit the growth of different human or murine tumor cells but not that of the untransformed cell lines Wi 38 and 3T3.

Using baby hamster kidney cells, we found that dibutyryl cAMP inhibits the growth of both normal BHK_{21} and transformed cells ($BHK_{21}Py$) but does not prevent the $BHK_{21}Py$ cell cultures from reaching the same saturation density observed in the absence of the nucleotide. Johnson and Pastan (6), working with 3T3 and SV 3T3, have recently made the same observations.

Furthermore, since we previously observed (1) an inhibition of the phosphate transport and metabolism in contact-inhibited cells, we have studied the effect of theophylline and dibutyryl cAMP on the phosphate transport and metabolism in normal and transformed cells. The possibility that cAMP may be an intermediate in the density-dependent inhibition of growth is discussed.

[1] The abbreviations used are: cAMP, 3',5'-cyclic adenosine monophosphate; dibutyryl cAMP, N^6, $O^{2'}$-dibutyryl 3',5'-cyclic adenosine monophosphate; P_o, organic phosphate.

Received July 7, 1972; accepted May 15, 1973.

MATERIALS AND METHODS

Dibutyryl cAMP from Sigma Chemical Co., St. Louis, Mo., contains no P_i and 2% of 2'-monobutyryl cyclic adenosine monophosphoric acid.

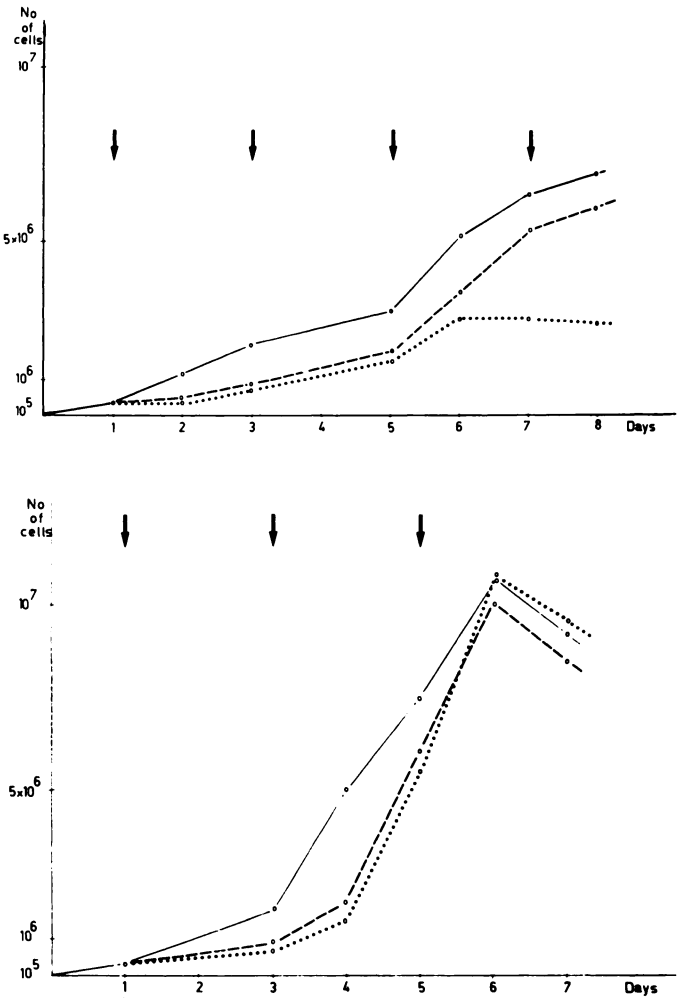
Cells and Growth Media. Baby hamster kidney cells $BHK_{21}C_{13}$ and $BHK_{21}Py$ (same cells transformed by polyoma virus) from Stoker's laboratories (11, 12) were judged free of *mycoplasma* by 2 different techniques. They were routinely cultured in 25-sq cm Falcon bottles in 4 ml BHK medium (12) supplemented with bicarbonate buffer, 10% Tryptose phosphate broth, and 10% calf serum.

Measurement of Phosphate Transport and Metabolism. Following incubation with ^{32}P, cells were washed with 0.9% NaCl solution and extracted with 1 ml 10% (w/v) perchloric acid at 0°. Acid-insoluble fraction was washed with ethanol (saturated with sodium acetate) and dissolved in 0.6 N NaOH at 37° for 24 hr. Protein was determined on aliquots of this fraction by the technique of Lowry *et al.* (9). The acid-soluble fraction (0.4 ml) was diluted to 1 ml with water, and fractionation of P_i and P_o was achieved by the butanol technique (13). Aliquots of acid-soluble P_i and P_o fractions were counted by the Cerenkov effect in a Beckman scintillator counter. Transport and metabolism of phosphate are expressed in cpm ^{32}P per 100 μg protein.

RESULTS

Inhibition of Cellular Growth by Theophylline and Theophylline plus Dibutyryl cAMP. Charts 1 and 2 show that the growth curves of $BHK_{21}C_{13}$ and $BHK_{21}Py$ are very different. Under these experimental conditions, transformed cells with no treatment grow to a higher cell density than normal cells without a plateau phase.

Addition of theophylline alone or theophylline plus dibutyryl cAMP to BHK_{21} (Chart 1) or $BHK_{21}Py$ cells (Chart 2) in exponential phase produces about 50% inhibition of the cell multiplication. Changing the medium every 48 hrs does not give a real plateau phase for untreated $BHK_{21}C_{13}$ cell cultures but, after 6 days, a net decrease in growth rate is observed. Addition of dibutyryl cAMP in the presence of theophylline gives a real plateau phase at a cell density lower than that observed in untreated cells or in those treated with theophylline alone. For $BHK_{21}Py$ cultures, addition of theophylline alone or theophylline plus



Charts 1 and 2. Effect of theophylline and dibutyryl cAMP plus theophylline on the growth of $BHK_{21}C_{13}$ (Chart 1) and $BHK_{21}Py$ cells (Chart 2). Cells were seeded at 10^5 cells/60-cu mm Falcon tissue culture dish. The additives were introduced in the medium 24 hr later. The medium with and without addition was changed every 2 days (*arrow*). O——O, culture cells without addition; O- - -O, plus theophylline (1 mM); O····O, plus theophylline (1 mM) + dibutyryl cAMP (1 mM). Arithmetical coordinates.

dibutyryl cAMP produces an inhibition of cell growth but does not prevent the cells from reaching the same high density observed in untreated cultures. Similar results were obtained in 3 different experiments. The growth inhibition of normal cells by dibutyryl cAMP was confirmed by using primary cultures of baby hamster kidney cells, in which cell multiplication is diminished by 50% after addition of the drug.

Effect of Theophylline and Dibutyryl cAMP on Phosphate Transport and Metabolism. In $BHK_{21}C_{13}$ cells, addition of theophylline alone, dibutyryl cAMP alone, or theophylline plus dibutyryl cAMP produces in all experiments an inhibition of phosphate transport, measured by radioactive P_i of the cells (Table 1). The inhibitions produced by theophylline and dibutyryl cAMP are not additive since dibutyryl cAMP alone gives the same inhibition as theophylline plus dibutyryl cAMP.

Radioactive P_o (of the acid-soluble fraction) (Table 2) is also decreased in the presence of the drugs. However, this decrease does not seem to be due only to inhibition of the phosphate transport; if so, the ratio P_o/P_i would be constant and the percentage of variation of P_o/P_i would be 0. In the presence of theophylline alone or dibutyryl cAMP plus theophylline, P_o is more decreased than P_i in 9 out of 10 experiments and the percentage of variation of P_o/P_i is negative (Table 3).

In the presence of dibutyryl cAMP alone the figure is different, as in some experiments P_o is more inhibited than P_i, while in others (Table 3, Experiments 5 and 7) P_i is more inhibited than P_o. Therefore the percentage of variation of P_o/P_i is positive. In $BHK_{21}Py$ cells, addition of the drugs does not produce an inhibition of phosphate transport in all experiments (Table 4). In particular, phosphate transport is not inhibited in 6 out of 6 experiments concerning dense cell cultures (Table 4, Experiments 5′, 6′, and 7′). In cells of cultures in exponential growth phase, phosphate transport is inhibited in 8 out of 10 experiments and radioactive P_o is decreased in the presence of the drugs in all experiments except one (Table 5).

In $BHK_{21}Py$ as for $BHK_{21}C_{13}$ cells, in the presence of theophylline or theophylline plus dibutyryl cAMP the percentage of variation of P_o/P_i (Table 6) is negative in 9 out of 10 experiments, which means that P_o is more inhibited than P_i. In the presence of dibutyryl cAMP alone, the percentage of variation of P_o/P_i is negative or positive according to the experiments.

DISCUSSION

In our experiments, theophylline alone, and theophylline plus dibutyryl cAMP, produced an inhibition of growth rate in $BHK_{21}C_{13}$ as well as in $BHK_{21}Py$ cells. Furthermore, dibutyryl cAMP plus theophylline decreased the saturation density of $BHK_{21}C_{13}$ cell cultures but not that of $BHK_{21}Py$ cells. These results differ from those reported by Heidrick and Ryan (4) and Sheppard (15), but agree with recent experiments of Johnson and Pastan (6).

The inhibition of growth rate may be explained by the inhibition of phosphate transport and metabolism, which we have observed both in normal and in transformed cells within 15 min after addition of dibutyryl cAMP or theophylline.

In most experiments, addition of the drugs produces a decrease in radioative P_i and P_o, but the latter does not seem to be only a result of the decrease of the phosphate transport (radioactive P_i). Theophylline, in particular, may act as an inhibitor of oxidative phosphorylation. Degradation products of dibutyryl cAMP do not seem responsible for the observed inhibition; furthermore the results of Table 7, show that butyrate or 5′-AMP does not produce the same inhibition as dibutyryl cAMP in $BHK_{21}C_{13}$.

If the decrease of growth rate may be explained by the inhibition of phosphate transport and metabolism, it is not clear why theophylline plus dibutyryl cAMP decreases the saturation density of normal cells and not that of transformed cells. However, the effect of dibutyryl cAMP plus

Table 1

Phosphate transport (radioactive P_i) in $BHK_{21}C_{13}$ cells

Duplicate cultures of $BHK_{21}C_{13}$ cells in log phase (Experiments 1 to 4) or in confluency (Experiments 5 to 7) were incubated for 15 min at 37° in 3.6 ml of fresh BHK medium (12), pH 7.4 supplemented with 28 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid instead of bicarbonate. ^{32}P (20 μCi/ml) and drugs were added in 0.4 ml of 0.9% NaCl solution at a concentration of 1 mM for both theophylline and dibutyryl cAMP. Radioactive P_i was measured on acid-soluble fraction and expressed in cpm ^{32}P per 100 μg proteins.

Percentage of variation: (cpm ^{32}P with the drug − cpm ^{32}P in control)/(cpm ^{32}P in control) × 100.

		+ theophylline		Theophylline + dibutyryl cAMP		+ dibutyryl cAMP	
Experiment	Control (cpm)	cpm	% variation	cpm	% variation	cpm	% variation
1	4,950	3,900	−20	2,440	−50	2,450	−50
2	6,400	4,030	−37	3,280	−49	3,580	−44
3	6,300					4,475	−30
4	5,400	4,100	−24	4,800	−11	4,660	−13
5	4.025	2,625	−35	2,250	−44	2,480	−38
6	4,950	4,300	−13	3,970	−20		
7	3,700					2,460	−34

Table 2

Phosphate metabolism (radioactive P_o) in $BHK_{21}C_{13}$ cells

Radioactive P_o of acid-soluble fraction corresponds to experiments of Table 1.

		+ theophylline		Theophylline + dibutyryl cAMP		+ dibutyryl cAMP	
Experiment	Control (cpm)	cpm	% variation	cpm	% variation	cpm	% variation
1	11,740	6,300	−46	5,470	−53	5,200	−55
2	12,200	6,800	−44	5,640	−53	7,120	−41
3	9,250					5,075	−45
4	10,000	6,540	−34	6,950	−30	6,860	−31
5	5,370	3,200	−40	3,070	−44	4,500	−16
6	5,100	4,420	−13	4,100	−19		
7	4,220					3,450	−18

Table 3

Variation of ^{32}P uptake and of P_o/P_i in $BHK_{21}C_{13}$ cells

Percentage of variation of acid-soluble fraction and of P_o/P_i corresponds to experiments of Tables 1 and 2.
Percentage of variation of acid-soluble fraction:

(cpm with the drug − cpm in control)/(cpm in control) × 100

Percentage of variation of P_o/P_i:

[(P_o/P_i with the drug − P_o/P_i in control)]/(P_o/P_i in control) × 100

	% variation of acid-soluble fraction			% variation of P_o/P_i		
Experiment	+ theophylline	Theophylline + dibutyryl cAMP	Dibutyryl cAMP	+ theophylline	Theophylline + dibutyryl cAMP	Dibutyryl cAMP
1	−34	−52	−55	−21	−8	0
2	−39	−45	−35	−10	−10	0
3			−34			−20
4	−32	−28	−25	−14	−20	−20
5	−38	−43	−30	−7	0	+37
6	−6	−17		−10	−10	
7			−23			+23
Mean	−30 ± 6[a]	−37 ± 6	−37 ± 5	−12 ± 2.5	−10 ± 3	

[a] Standard deviation.

Table 4

Phosphate transport (radioactive P_i) in $BHK_{21}Py$ cells

Same kind experiments as in Table 1, but with $BHK_{21}Py$ cells instead of $BHK_{21}C_{13}$ cells. In Experiments 1′ to 4′ cultures are in log phase and in Experiments 5′ to 7′ they are in confluency. Radioactive P_i and P_o were measured on acid-soluble fraction and expressed in cpm ^{32}P per 100 μg proteins.

		+ theophylline		Theophylline + dibutyryl cAMP		+ dibutyryl cAMP	
Experiment	Control (cpm)	cpm	% variation	cpm	% variation	cpm	% variation
1′	8,075	5,740	−28	5,115	−37	5,155	−36
2′	6,225	7,250	+16	4,830	−22	6,250	0
3′	8,410					6,400	−24
4′	6,030	4,540	−25	5,000	−16	3,980	−34
5′	4,040	4,000	0	5,300	+31	4,990	+23
6′	5,570					5,940	+7
7′	3,000	3,175	+6	3,380	+10		

Table 5

Phosphate metabolism (radioactive P_o) in $BHK_{21}Py$ cells

Radioactive P_o of acid-soluble fraction corresponds to experiments of Table 4.

		+ theophylline		Theophylline + dibutyryl cAMP		+ dibutyryl cAMP	
Experiment	Control (cpm)	cpm	% variation	cpm	% variation	cpm	% variation
1′	11,850	7,925	−33	7,580	−36	11,000	−7
2′	14,700	14,000	−5	7,600	−48	12,600	−14
3′	9,625					10,450	+8
4′	10,000	6,115	−38	7,070	−29	6,050	−40
5′	8,380	6,100	−27	6,750	−20	5,425	−35
6′	5,450					4,780	−12
7′	3,650	2,635	−27	3,350	−8		

Table 6

Variation of ^{32}P uptake and of P_o/P_i in $BHK_{21}Py$ cells

Percentage of variation of acid-soluble fraction and of P_o/P_i corresponds to experiments of Tables 4 and 5.

	% variation of acid-soluble ^{32}P			% variation of P_o/P_i		
Experiment	+ theophylline	theophylline + dibutyryl cAMP	Dibutyryl cAMP	+ theophylline	Theophylline + dibutyryl cAMP	Dibutyryl cAMP
1′	−26	−30	−21	−8	0	+40
2′	+8	−42	0	−15	−30	−17
3′			−18			+43
4′	−32	−20	−33	−20	−15	−10
5′	−15	5	−6	−28	−38	−48
6′			−13			−20
7′	−13	0		−33	−18	
Mean	−16 ± 7[a]	−20 ± 8	−15 ± 5	−21 ± 4.5	−20 ± 6.5	

[a] Standard deviation.

Table 7

	Acid-soluble cpm/100 μg protein	% variation	P_i	% variation	P_o	% variation
Control	24,200		7,400		17,400	
Dibutyryl cAMP (10^{-3} M)	20,100	−17	5,800	−22	15,000	−14
5′-AMP (10^{-3} M)	39,200	+62	7,750	+5	32,000	+84
Control	12,750		4,690		8,260	
Dibutyryl cAMP (10^{-3} M)	8,250	−33	2,675	−42	5,550	−32
Butyrate (10^{-4} M)	14,100	+10	4,050	−13	9,650	+17

theophylline on the phosphate uptake is, in the mean, lower in $BHK_{21}Py$ cells than in $BHK_{21}C_{13}$ cells, specifically in high-cell-density cultures (Table 3, Experiments 5 to 7; Table 6, Experiments 5′ to 7′).

The concentration of dibutyryl cAMP used (10^{-3} M) may seem rather high and it would be so if dibutyryl cAMP acts on the outside of the cell membrane. If dibutyryl cAMP acts inside the cell, after 15 min incubation, the concentration used gives intracellular condition very close to the physiological one. According to the paper of Kaukel and Hilz (8), after incubating HeLa cells for 45 min in a medium containing 10^{-3} M dibutyryl cAMP, only 10 pmoles dibutyryl cAMP and 15 pmoles 2′-monobutyryl cAMP were found per 10^6 cells. These values are similar to the intra-cellular concentration of cAMP (16 pmoles/10^6 cells) found by Heidrick and Ryan in HeLa cell culture at saturation density (5). When cells are actively growing, the concentration of cAMP is lower (5, 14). It has been shown in lymphocytes that cAMP in concentrations higher than 10^{-5} M induces an inhibition of DNA synthesis and of mitotic activity and that, on the contrary, cAMP in concentration 10^{-6} to 10^{-8} M enhances DNA synthesis (3, 10).

The modulation of cAMP concentration in the cells would be responsible for active growth or for growth inhibition of the cells. Our results agree with this hypothesis. We show that dibutyryl cAMP induces a decrease in the phosphate transport and metabolism in cells. We have observed (1), and this has been confirmed (2), that both transport and metabolism of phosphate decrease when the culture cells enter the stationary growth phase. Addition of serum to stationary cultures enhances phosphate transport and metabolism, (2, 7) and recently it has been shown that, after 10 min, addition of serum decreases cAMP concentration in the cells (14). This result would explain the opposite effect of (10^{-3} M) dibutyryl cAMP and serum (10%) on phosphate transport and metabolism in the cells.

If cAMP is an intermediate in the contact inhibition of growth, a difference in the sensitivity toward this nucleotide might explain why the cultures of BHK-transformed cells are less "contact inhibited" and can reach a higher cell density than those of normal cells.

REFERENCES

1. Blade, E., Harel, L., and Hanania, N. Variation du Taux d'Incorporation du Phosphore dans les Cellules en Fonction de Leurs Concentrations et Inhibition de Contact. Exptl. Cell Res., *41:* 473–482, 1966.
2. Cunningham, D. D., and Pardee, A. B. Transport Changes Rapidly Initiated by Serum Addition to "Contact Inhibited" 3T3 Cells. Proc. Natl. Acad. Sci. U. S., *64:* 1049–1056, 1969.
3. Gallo, R. C., and Whang-Peng J. Enhanced Transformation of Human Immunocompetent Cells by Dibutyryl Adenosine Cyclic 3′,5′-Monophosphate. J. Natl. Cancer Inst., *47:* 91–94, 1971.
4. Heidrick, M. L., and Ryan, W. L. Cyclic Nucleotides on Cell Growth *in Vitro*. Cancer Res., *30:* 376–378, 1970.
5. Heidrick, M. L., and Ryan, W. L. Adenosine 3′,5′-Cyclic Monophosphate and Contact Inhibition. Cancer Res., *31:* 1313–1315, 1971.
6. Johnson, G. S., and Pastan, I. Role of 3′,5′-Adenosine Monophosphate in Regulation of Morphology and Growth of Transformed and Normal Fibroblasts. J. Natl. Cancer Inst., *48:* 1377–1383, 1972.
7. Jullien, M., Blat, C., and Harel, L. Effect of pH and Serum on Phosphate Transport in BHK_{21} and BHK_{21} Polyoma Transformed Cells. Exptl. Cell Res., *73:* 530–533, 1972.
8. Kaukel, E., and Hilz, H. Permeation of Dibutyryl cAMP into HeLa Cells and Its Conversion to Monobutyryl cAMP. Biochem. Biophys. Res. Commun., *46:* 1011–1018, 1972.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., *193:* 265–275, 1951.
10. MacManus, J. P., and Whitfield, J. F. Stimulation of DNA Synthesis and Mitotic Activity of Thymic Lymphocytes by Cyclic Adenosine 3′5′-Monophosphate. Exptl. Cell Res., *58:* 188–191, 1969.
11. Mac Pherson, I. Characteristics of a Hamster Cell Clone Transformed by Polyoma Virus. J. Natl. Cancer Inst., *30:* 795–806, 1963.
12. Mac Pherson, I., and Stocker, M. Polyoma Transformation of Hamster Cell Clones—An Investigation of Genetic Factors Affecting Cell Competence. Virology, *16:* 147–151, 1962.
13. Marsh, B. B. The Estimation of Inorganic Phosphate in the Presence of Adenosine Triphosphate. Biochim. Biophys. Acta, *32:* 357–361, 1959.
14. Otten, J., Johnson, G. S., and Pastan, I. Regulation of Cell Growth by Cyclic Adenosine 3′,5′-Monophosphate. J. Biol. Chem., *247:* 7082–7087, 1972.
15. Sheppard, J. R. Restoration of Contact-inhibited Growth to Transformed Cells by Dibutyryl Adenosine 3′:5′-Cyclic Monophosphate. Proc. Natl. Acad. Sci. U. S., *68:* 1316–1320, 1971.