EFFECT OF ADENOSINE 3′-5′-CYCLIC MONOPHOSPHATE ON CELL PROLIFERATION

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ABSTRACT

Secondary cultures of human diploid fibroblasts, which demonstrate density-dependent inhibition of cell growth, were used to study the effect of adenosine 3′-5′-cyclic monophosphate (cAMP) on cell proliferation. DNA synthesis in nonconfluent cultures and in contact-inhibited cultures stimulated to grow by refeeding with fresh medium was found to be inhibited by exogenous cAMP. The properties of this inhibition of DNA synthesis, together with the alterations in cAMP metabolism observed in confluent cultures of cells stimulated with fresh medium to resume growth, strongly suggest that cAMP is involved in contact-inhibition of cell proliferation.

INTRODUCTION

Several studies suggest a possible inhibitory role for adenosine 3′,5′-cyclic monophosphate (cAMP) in the regulation of cellular proliferation in fibroblast cultures. Cells of malignant origin contain either very low or no membrane adenyl cyclase activity (11, 17); and baby hamster kidney (BHK) cells transformed by polyoma virus appear to have less adenyl cyclase activity than the untransformed parental cell line (1). In addition, the proliferation of L cells and HeLa cells (26) is inhibited by exogenous cAMP. Recently N^6, O^2-dibutyryl-cAMP (db-cAMP) has been shown to restore normal growth characteristics to cells from a Rous sarcoma virus-induced rat sarcoma (13) and to 3T3 cells which were transformed either spontaneously or by polyoma virus (29). Finally, cAMP levels in normal 3T3 cells have been found to be higher than those found in transformed 3T3 cells (20, 30).

The aim of these studies is to further characterize the effects of cAMP on cell growth and to determine whether cAMP is involved in the density-dependent inhibition of cellular proliferation observed in secondary cultures of human diploid fibroblasts.

MATERIALS AND METHODS

Cell Cultures

Strains of human diploid fibroblasts obtained from skin biopsies were supplied by Dr. Henry Nadler. Standard tissue culture procedures were used and no stain was carried in culture for more than 3 or 4 wk. All cells were grown on 60 mm or 100 mm plastic tissue culture Petri dishes (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.). The culture medium, Eagle's Minimal Essential Medium (EMEM) (4) supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) unless otherwise indicated, was changed every 2–3 days and always 2 days before an experiment was started. Theophylline, a gift of Dr. Oscar Hechter, db-cAMP (Sigma Chemical Co., St. Louis, Mo.), or cAMP (Sigma) was added to the culture medium when the effects of these compounds on cell growth were being studied. The sodium salts of both cAMP and db-cAMP were used exclusively.

Incorporation Studies with Tritiated Thymidine

Uptake of thymidine-^{3}H (TdR-^{3}H) into acid-precipitable material was used to measure the rate of DNA synthesis, and therefore the proliferative activity, in cell cultures. Cultures were incubated for 30 min in 2.0 ml of culture medium containing 2.0 μCi of TdR-^{3}H (New England Nuclear Corp. (Boston, Mass.); specific activity, 6 Ci/mmole). After 30 min, the medium was discarded and the cells were washed three times with cold 0.15 M NaCl. Each culture was then dissolved in a known amount of 1.0 N NaOH. A sample of this was used to determine the protein content of the culture using the method of Lowry (16), while the remainder was neutralized with 1.0 N HCl and precipitated with trichloroacetic acid (TCA) at a final concentration of 5%. The precipitate, after being washed three times with 5% TCA, was dissolved in 1.0 ml of 0.05 N NaOH, and 0.1 ml was counted in a Packard Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) with an efficiency of 15%. The rate of DNA synthesis was always expressed as TCA-precipitable cpm/mg cell protein, and in some experiments this rate was then normalized to be a percentage of an appropriate control value.

Radioimmunoassay for Cyclic AMP

A radioimmunoassay was used to measure cAMP (32). ^{125}I-succinyl-cAMP-tyrosinemethylester (^{125}I-ScAMP-TME) and rabbit anti-cAMP serum were purchased from Collaborative Research Inc., Waltham, Mass. The goat anti-rabbit IgG serum was purchased from Miles Laboratories, Inc., Elkhart, Ind.

Extracts of cultures for which cAMP content was to be determined were prepared in the following manner. After pouring off the medium used in an experiment, the cultures, without being washed, were frozen by floating the Petri dishes on an acetone-dry ice bath for 5–10 sec. The cultures were then overlaid with 1.0–1.5 ml of 6% TCA, and the cells, after thawing, were scraped off the dishes with a rubber policeman. After centrifugation at 4000 rpm for 15 min, each supernatant was extracted five times with 4–5 ml of petroleum ether saturated with water. The aqueous phase was heated at 80°C for 2 min and then evaporated to dryness. The residue was dissolved in 0.05 M Na acetate buffer, pH 6.2, and used directly in the immunoassay.

Each assay tube contained either 50–300 μl of tissue extract or 0.5–100 pmoles of a standard solution of cAMP made up in 0.05 M Na acetate buffer, pH 6.2. To this was added 0.1 ml of anti-cAMP serum diluted 1 to 300 in the acetate buffer and 0.05 ml of the ^{125}I-ScAMP-TME. A final volume of 0.5 ml was obtained by addition of 0.05 M Na acetate buffer, pH 6.2. The tubes were incubated for 2–4 hr at 4°C; 0.03 ml of goat anti-rabbit IgG was then added, and the incubation was continued overnight. The precipitates were collected by centrifugation at 4000 rpm at 4°C for 20 min, washed once with 1.0 ml of cold Na acetate buffer, and counted in a Packard Auto-Gamma Spectrometer. Analyses were carried out in duplicate.

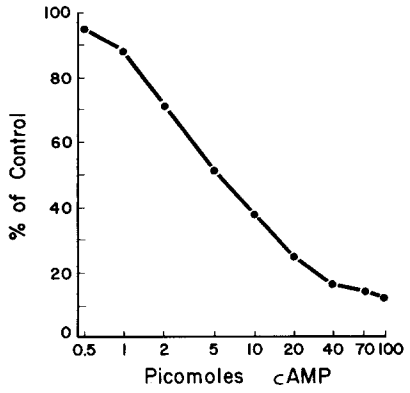

FIGURE 1 Standard curve for determination of cAMP. Radioimmunoassays were performed as described in Materials and Methods. The amount of radioactive ^{125}I-ScAMP-TME precipitated in the presence of 0.5–100 pmoles of cAMP was expressed as a percentage of that precipitated in control assay tubes containing no unlabeled cAMP. Each point, representing the average of duplicate determinations, was plotted as a function of the amount of cAMP added to the assay.

Fig. 1 shows the standard curve for the displacement of ^{125}I-ScAMP-TME from the immunoprecipitates by known amounts of unlabeled cAMP. When plotted as a semilogarithmic function of unlabeled cAMP added to the assay, the amount of ^{125}I-ScAMP-TME bound in the precipitates, expressed as a percentage of that bound in precipitates from control assay tubes to which no unlabeled cAMP had been added, decreased linearly in the range of 1.0–20.0 pmoles. For each culture extract, the amount of ^{125}I-ScAMP-TME precipitated when a sample of extract was used in the assay was expressed as a percentage of that precipitated in control tubes which contained no added extract or unlabeled cAMP. With this percentage, the cAMP content of that sample was then determined directly from the standard curve.

The following controls were run to check the specificity of the assay for cAMP. First, because the cells were not washed before extracts were prepared, some medium, approximately 0.1–0.2 ml, was left on the cells, and was therefore extracted along with the cells. However, extracts prepared from as much as

1 ml of fresh EMEM containing 10% serum or from 1 ml of medium incubated on confluent cultures for 2 days contained no substances which affected the binding of ^{125}I-ScAMP-TME in the immunoassay. In addition, treatment of any culture extract with cAMP phosphodiesterase completely destroyed the ability of that extract to inhibit the precipitation of ^{125}I-ScAMP-TME. Finally, 1.4 mM theophylline, the highest concentration used in the experiments to be described here, had no effect in the assay. Thus, the assay appeared to be specific for cAMP.

In this study, all cAMP levels were expressed as pmoles cAMP/mg protein. Because the cultures used for determining cAMP content were not washed before being extracted, the protein content was measured in separate cultures by the same method employed to determine the protein content of cultures in pulse-labeling experiments.

RESULTS

Effect of Culture Density on DNA Synthesis

The effect of culture density on DNA synthesis was studied to determine the extent to which density-dependent inhibition of cell proliferation is manifested by secondary cultures of diploid human fibroblasts. Cultures seeded at widely varying densities were allowed to grow for several days. 2 days after their last feeding, DNA synthesis was determined as described in Materials and Methods.

The results, plotted as a function of cell protein density and expressed as a percentage of the synthesis found for a lightly seeded, nonconfluent culture which synthesized DNA at a maximum rate, demonstrate that DNA synthesis decreased drastically at densities greater than 30 μg protein/cm^2 of culture dish (Fig. 2). At densities greater than 50 μg/cm^2, synthesis was reduced to about 10% of that found in a growing, nonconfluent culture. These results are consistent with previously reported decreases in DNA synthesis observed at increasing densities of 3T3 cells (36) and human diploid fibroblast cultures (15).

Release from Growth Inhibition

The following experiment was performed in an attempt to reverse the inhibition of DNA synthesis observed in cell cultures at confluence. Contact-inhibited cultures, those with protein densities greater than 50 μg/cm^2, were refed, 2 days after their last medium change, with fresh EMEM

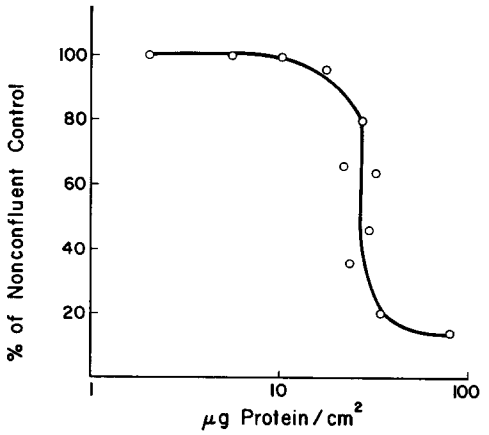

FIGURE 2 Effect of culture density on DNA synthesis. Cultures of cells seeded at widely varying densities were pulsed with TdR-^{3}H 2 days after their last medium change. Each value of DNA synthesis obtained was expressed as a percentage of that determined for a nonconfluent culture with a protein density of 10 μg/cm^2 which synthesized DNA at a maximal rate. Each point in the figure, plotted as a function of cell protein density, represents synthesis in a single culture.

containing 10% fetal calf serum. DNA synthesis was then followed as a function of time after refeeding.

In Fig. 3, it can be seen that such treatment resulted in a synchronous wave of DNA synthesis beginning 10–15 hr and peaking 20–25 hr after refeeding. These data indicate that at least some of the cells were released from the growth inhibitory effects of confluence and were allowed to initiate DNA synthesis simply by changing the medium. Moreover, since it has been demonstrated that the growth of normal fibroblasts is arrested in the G_1 phase of the mitotic cycle at large culture densities (19), the 10–15 hr interval between the medium change and the onset of DNA synthesis is consistent with an amount of time needed for those cells released from contact-inhibition to progress through the G_1 phase in a fairly synchronous fashion and enter S phase. Radioautographic experiments not reported here indicated that the number of cells stimulated to resume growth after a medium change represented about 10% of the total number of cells in a contact-inhibited culture.

This partial release from growth inhibition by refeeding has been demonstrated before (34, 36) and is thought to be due to a macromolecular substance in fresh serum which desensitizes a small

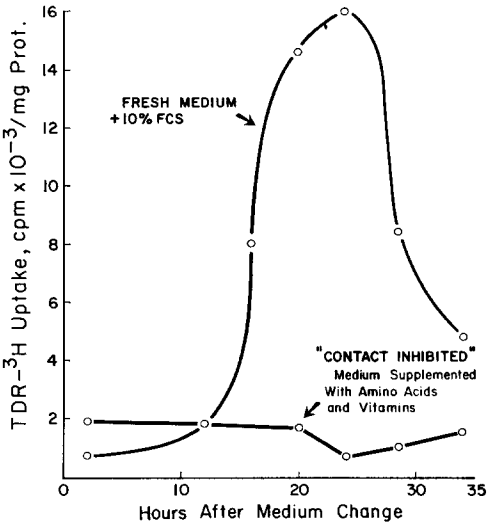

FIGURE 3 Release from growth inhibition. DNA synthesis was determined in confluent cultures as a function of time after being refed with fresh medium containing 10% fetal calf serum (FCS) or with contact-inhibited medium supplemented with amino acids and vitamins. Each point is the average of duplicate determinations.

percentage of cells to the inhibitory effects of cell contact (34). The macromolecule involved appears to be depleted from fresh medium after incubation on confluent cultures for 2 days, as such depleted medium (contact-inhibited medium) had no stimulatory effect on confluent cultures, even when replenished with vitamins and amino acids (Fig. 3). Therefore, to eliminate any residual stimulatory effect from the last medium change, all experiments described hereafter involving stimulation of contact-inhibited cells were performed on confluent cultures refed 2 days before.

Other results not shown here indicated that a 15-hr or 30-hr incubation with contact-inhibited medium had no inhibitory effect on DNA synthesis in nonconfluent cultures. Because it supports the proliferation of nonconfluent cells, the inability of contact-inhibited medium to stimulate confluent cultures was probably not due to the presence of toxic substances or the absence of nutrients essential for growth. Instead, these results are consistent with the idea that contact-inhibited medium is deficient in a labile serum factor which is necessary for overcoming the growth-inhibitory effects of cell contact. In nonconfluent cultures, where cellular interaction is not a great consideration, this serum factor would not be required for cell growth.

Thus, the experiment in Fig. 3 offers a system in which the transition from a contact-inhibited state to a growing state can be studied. With such studies, one might be able to gain information concerning the nature of the processes responsible for the inhibition of cell growth in the contact-inhibited state.

Inhibition of the Stimulation of DNA Synthesis After Refeeding Confluent Cultures

The effect on DNA synthesis of adding increasing concentrations of cAMP, db-cAMP, or

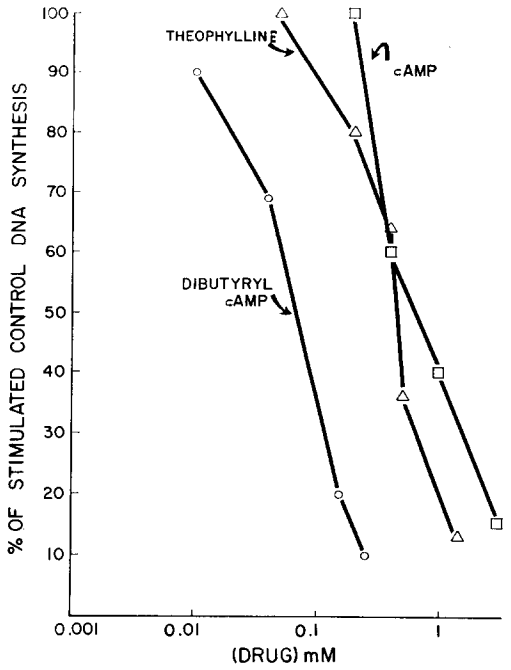

FIGURE 4 Effect of increasing concentrations of theophylline, db-cAMP, and cAMP on the stimulation of DNA synthesis after a medium change. Confluent cultures were refed with fresh medium containing increasing concentrations of theophylline, db-cAMP, or cAMP. Cultures refed with fresh medium containing none of these compounds served as controls. DNA synthesis in all cultures was determined after 20 hr, expressed as a percentage of the value for the stimulated controls, and plotted as a function of the millimolar concentration of drug present at the time of refeeding. Each point is the average of two or three determinations.

TABLE I

Effect of 0.08 mM db-cAMP or 0.6 mM Theophylline on DNA Synthesis in Confluent Cultures Refed with Fresh Medium

Time After Refeeding	DNA synthesis (cpm/mg protein)		
	Control	A	B
Hr			
0	2200	—	—
10	1500	1800	1200
15	6200	3700	3100
19	9600	6000	4200
23	11400	6700	5000
37	7200	4000	3800
35	4000	3100	2800

2 days after their last medium change, confluent cultures were refed with fresh medium containing 0.6 mM theophylline (A) or 0.08 mM db-cAMP (B). DNA synthesis was then followed, as described in Materials and Methods, as a function of time after refeeding. DNA synthesis was also followed in stimulated but otherwise untreated cultures (control group). Each value above is the average of two separate determinations.

theophylline, a cAMP phosphodiesterase inhibitor, to confluent cultures for the entire period of stimulation was studied. DNA synthesis was determined 20 hr after refeeding with fresh medium containing one of these compounds.

The results in Fig. 4, expressed as a percentage of the synthesis found for stimulated but otherwise untreated control cultures, indicate that 3.0 mM cAMP, 0.20 mM db-cAMP, or 1.4 mM theophylline completely inhibited the stimulation of DNA synthesis normally seen at 20 hr. The residual DNA synthesis seen with these concentrations of inhibitors was equivalent to that determined for unstimulated cultures of contact-inhibited cells. That much less db-cAMP was needed for inhibition is consistent with its possibly greater ability to enter cells and its relatively greater resistance to the cAMP phosphodiesterase (18, 22).

An additional experiment, shown in Table I, indicated that 20–25 hr represented the interval of maximum DNA synthesis after stimulation, as in the control group, even if 0.08 mM db-cAMP or 0.6 mM theophylline was present for the entire period after refeeding. Hence, for the experiment reported in Fig. 4, DNA synthesis only had to be measured at a single time, 20 hr, in order to determine the amount of stimulation relative to the controls.

Although commercial preparations of db-cAMP often contain considerable quantities of butyric acid, 1.0 mM sodium butyrate did not affect the stimulation of DNA synthesis after a medium change on confluent cultures. Thus, the effect of db-cAMP on DNA synthesis was probably not due to contamination with butyric acid. On the other hand, while adenosine and 5′-AMP caused virtually no inhibition of DNA synthesis in stimulated confluent cultures at concentrations below 2 mM, 3 mM adenosine or 5′-AMP caused 20–30% inhibition. Since 3 mM cAMP is a huge amount to add to cells in order to attain complete inhibition of DNA synthesis, any experiment which would require such a high concentration of cAMP was also performed with db-cAMP.

Effect of cAMP on Events Occurring Early After Stimulation of Confluent Cultures with Fresh Medium

The fact that 1.4 mM theophylline, 3.0 mM cAMP, or 0.20 mM db-cAMP completely inhibited the stimulation of DNA synthesis in confluent cultures after a medium change indicates that the growth of the cells which were released from contact-inhibition must have been arrested by these agents at some point before S phase. Additional investigations were carried out to demonstrate the reversible nature of this arrest of growth by cAMP and to determine the earliest time after stimulation at which it can occur.

In three separate experiments, maximally inhibitory quantities of cAMP, db-cAMP, or theophylline were added to confluent cultures at the time of stimulation. Other stimulated cultures served as controls. After a given period of stimulation in the presence of inhibitors, the drug-treated cultures were refed with fresh medium without inhibitors. DNA synthesis was then determined in both drug-treated and control cultures as a function of time after the initial stimulation.

The results in Fig. 5 demonstrate that treatment with 1.4 mM theophylline in the interval 0–4 hr after stimulation delayed the onset of DNA synthesis by 4 hr with respect to the onset of synthesis in the untreated controls, while treatment in the interval 0–8 hr delayed the onset of synthesis by 8 hr. Similarly, treatment with 0.25 mM db-

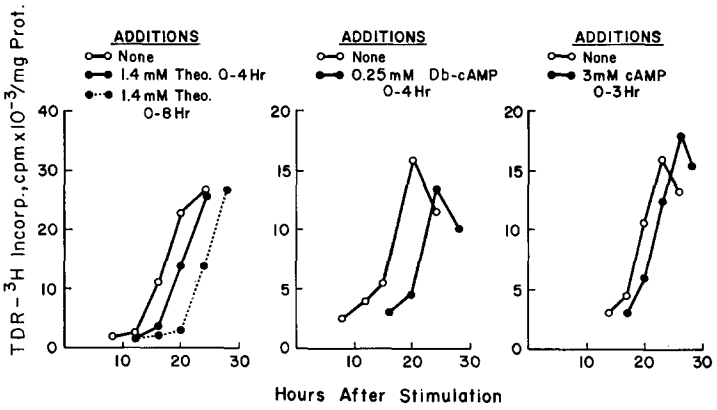

FIGURE 5 Effect of treatment with theophylline, db-cAMP, or cAMP during the early hours after stimulation on the initiation of DNA synthesis in confluent cultures. In three separate experiments, confluent cultures of cells were initially stimulated with fresh medium containing 1.4 mM theophylline (theo), 0.25 mM db-cAMP, or 3.0 mM cAMP. After a period of time in the presence of one of the inhibitors, the cultures were refed with fresh medium without inhibitors. DNA synthesis was then followed as a function of time after the initial stimulation. DNA synthesis was also followed in stimulated, but otherwise untreated cultures (open circles). Each point represents the average of two determinations.

cAMP in the interval 0–4 hr delayed the onset of DNA synthesis by 4 hr. Finally, treatment with 3.0 mM cAMP for 3 hr delayed the onset of DNA synthesis by 3 hr.

Since the delay in DNA synthesis was equal to the amount of time each inhibitor was present after stimulation, it can be concluded that the early events necessary for progression through G_1 and entry into S phase were inhibited by cAMP. This conclusion is consistent with the hypothesis that the stimulation of growth normally seen after refeeding confluent cultures with fresh medium is due to a lowering of the intracellular cAMP level, thereby allowing these critical early events to occur. Thus, the growth-stimulatory action of fresh serum may be to decrease cAMP levels.

Effect of cAMP on Nonconfluent Cells

The possibility that high levels of cAMP might be necessary for the inhibition of cell growth in confluent cultures of normal fibroblasts led to studies involving the effect of cAMP on nonconfluent cells. Fig. 6 shows that the rate of DNA synthesis in nonconfluent cultures decreased in a time-dependent fashion during a 25 hr incubation with medium containing 1.4 mM theophylline. After 25 hr, synthesis was found to be reduced to 20% of the control level. An additional experiment, not shown here, demonstrated that a treatment of 20–30 hr duration was also required for any appreciable inhibition of DNA synthesis in sparsely populated cultures by 0.2 mM db-cAMP. These results indicate that DNA synthesis in nonconfluent cells can be inhibited by fairly lengthy treatments with exogenous cAMP.

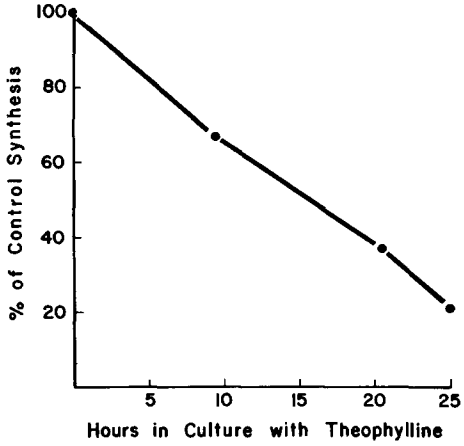

FIGURE 6 Effect of 1.4 mM theophylline on DNA synthesis in nonconfluent cells. Nonconfluent cultures were treated with 1.4 mM theophylline. At various times after treatment, DNA synthesis was determined as described in Materials and Methods and was expressed as a percentage of the synthesis determined for control cultures not treated with theophylline. Each point is the average of the synthesis in duplicate cultures. At the time of pulsing with TdR-^{3}H, no culture contained more than 8 μg protein/cm^2 of culture dish.

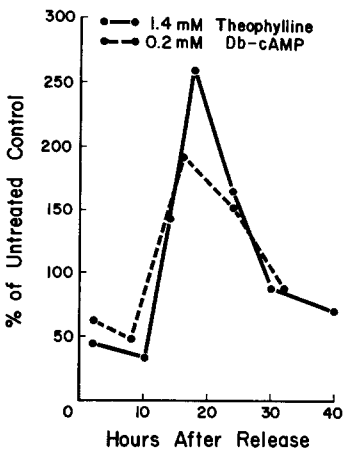

FIGURE 7 Reversal of the inhibitory effect of 1.4 mM theophylline or 0.20 mM db-cAMP on DNA synthesis in nonconfluent cultures. Nonconfluent cultures were treated with either 1.4 mM theophylline or 0.20 mM db-cAMP. Untreated cultures served as controls. After 30 hr, all cultures, including controls, were refed with fresh medium containing no inhibitor. DNA synthesis in the control cultures was determined at a single time, 2 hr after refeeding. Only this determination was necessary to establish a control or 100% value because the synthesis in untreated cultures was found to be independent of culture density below 10 μg prot./cm^2 (Fig. 2). DNA synthesis in the drug-treated cultures, however, was followed as a complete function of time after the removal of the inhibitors; and each point, representing the average of two similarly treated cultures, was expressed as a percentage of the synthesis determined for the controls.

An experiment was performed next to determine if the inhibition of DNA synthesis described in Fig. 6 was due to an arrest of cell growth in the G_1 phase of the cell cycle. Two groups of nonconfluent cultures were treated with 1.4 mM theophylline or 0.20 mM db-cAMP for 30 hr. Cultures serving as controls remained untreated. After 30 hr, all cultures, including controls, were refed with fresh medium without inhibitors. DNA synthesis in the control group was determined at a single time, 2 hr after this medium change. In the two drug-treated groups, however, synthesis was followed as a complete function of time after the removal of the inhibitors. At no time during the entire course of this experiment did any culture reach a protein density greater than 9 μg cell protein/cm^2.

The results in Fig. 7, expressed as a percentage of the synthesis determined for the untreated controls, indicate that a 30 hr treatment of nonconfluent cultures with 1.4 mM theophylline or 0.20 mM db-cAMP decreased DNA synthesis by 60% and 40%, respectively. Furthermore, synchronous waves of DNA synthesis, containing peak values 1.9–2.6 times the control level of synthesis, occurred in the interval 10–30 hr after the removal of the inhibitors. This pattern of synthesis, which includes a 10 hr lag period before the synchronous onset of S phase, is very similar to that seen in Fig. 3 in which confluent cultures, arrested in G_1, were released from growth inhibition by refeeding. Hence, cAMP seems to inhibit DNA synthesis in nonconfluent cultures by blocking cellular events occurring early in G_1 which are necessary for progression through that phase and entry into S phase.

Measurement of cAMP After Stimulation of Confluent Cultures

In one experiment, cultures were grown to confluence in EMEM containing only 1.3% serum. After contact-inhibition was achieved in this medium at a culture density which was low compared to the saturation densities reached in 10% serum (data not shown), stimulation was carried out with EMEM containing 10% serum. Experiments not reported here demonstrated that such treatment resulted in a 30% increase in cell protein after 40 hr of stimulation, while stimulation of cultures grown to higher saturation densities in the usual medium containing 10% serum resulted in only a 10% increase in cell protein after 40 hr. This agrees with other studies (7, 27) which have indicated that growing cells to confluence in a concentration of serum much lower than that used for stimulation increases the percentage of cells released from contact-inhibition.

cAMP was determined as a function of time after stimulation as described in Materials and Methods. Duplicate sets of cultures were stimulated to monitor changes in protein content; and in any culture the cAMP level was expressed as pmoles cAMP/mg protein. The average value of cAMP content for any time after stimulation was then normalized to be a percentage of that observed for contact-inhibited, unstimulated control cultures.

The results in Table II indicate that cAMP levels decreased by 60% within the first hour after stimulation and remained low at 2, 5, and 10 hr. These data are consistent with the hypothesis that

TABLE II

Changes in cAMP Content after Stimulation

Time after stimulation	cAMP Content (pmoles/mg protein)	Mean (SD)	Control mean
Hr			%
Unstimulated controls	12.5 6.5 9.8 10.4	9.8 (2.0)	100
1	3.8 3.5 4.5	3.9 (0.4)	40
2	3.8 4.4	4.1 (0.3)	42
5	4.4 4.8 3.7	4.3 (0.5)	44
10	4.4 3.9	4.2 (0.25)	43

Cultures grown to confluence in EMEM containing 1.3% serum were stimulated with EMEM containing 10% serum. Unstimulated cultures served as controls. cAMP determinations were made as a function of time after stimulation as described in Materials and Methods. Individual values of cAMP content as well as the average value are reported for each time period. The standard deviation from the mean "SD = $\left(\frac{\Sigma (x-\bar{x})^2}{N}\right)^{1/2}$ is given in parentheses next to the mean. In this calculation, X is any one of the N separately determined values averaged to obtain the mean value, $\bar{X}$.

stimulation of DNA synthesis in contact-inhibited cells is due to a decrease in cAMP levels.

An additional experiment was performed to study further the relationship between alterations in cAMP metabolism and stimulation of growth. Table III demonstrates cAMP levels in cultures grown to confluence in EMEM containing 10% serum and stimulated with the same medium. The 36% decrease in cAMP levels observed 2 hr after stimulation of such cultures was smaller than the 58% decrease found 2 hr after stimulation of cultures grown to confluence in 1.3% serum (Table II). This is consistent with the previously mentioned smaller percentage increase in cell protein 40 hr after stimulation of cultures grown up in 10% serum. The percentage decrease in cAMP levels thus correlates with the percentage stimulation of cell growth.

It was previously shown that 1.4 mM theophylline was required to inhibit completely the stimulation of DNA synthesis after a medium change (Fig. 4). Table III demonstrates that this same concentration also inhibited completely the 36% decrease in cAMP levels, maintaining them at the value found for the unstimulated controls. If the primary effect of theophylline on growth is due to its action on cAMP metabolism, then these data strongly suggest that the transition from a contact-inhibited state to a growing state is due to a decrease in the intracellular level of cAMP caused by refeeding with fresh medium.

Potentiation of cAMP Action by Theophylline

If theophylline inhibits proliferation primarily through its effect on the cAMP phosphodiesterase,

TABLE III

Effect of Stimulation on cAMP Levels in Confluent Cultures

Group	cAMP Content (pmoles/mg protein)	Mean (SD)	Control mean
			%
Unstimulated controls	9.9 10.4 9.6	10.0 (0.32)	100
A	7.4 5.3 6.4	6.4 (0.82)	64
B	10.1 11.5 10.8	10.8 (0.6)	108

Cultures grown to confluence in EMEM containing 10% fetal calf serum were stimulated with that same medium for 2 hr in the absence (A) or presence (B) of 1.4 mM theophylline. Unstimulated cultures served as controls. After 2 hr, cAMP content was determined as described in Materials and Methods. The standard deviation from the mean, as defined in the legend to Table II, is given in parentheses next to the average for each group.

TABLE IV

Potentiation of the Inhibitory Effect of cAMP by Theophylline

Group	Control DNA Synthesis (s.d.)	Inhibition of control synthesis
	%	%
Control	100 (1.7)	0
A	93 (3.2)	7
B	97 (5.4)	3
C	67 (4.1)	33

In three separate though identical experiments, four groups of two or three nonconfluent cultures each were refed with fresh medium containing no additions (control), 0.225 mM cAMP (A), 0.45 mM theophylline (B), or both 0.225 mM cAMP and 0.45 mM theophylline (C). DNA synthesis in the four groups was determined 24 hr later as described in Materials and Methods. In each separate experiment, the average value for DNA synthesis in the control group was determined. All individual values for DNA synthesis in that experiment, including those which were averaged to obtain the control mean, were expressed as a percentage of that mean. These normalized data from the three separate experiments were then combined according to group. The average for each group of pooled results is given above followed by the standard deviation from the mean in parentheses. At no time in these experiments did the protein density of any culture exceed 7 $\mu g/cm^2$.

then a concentration of theophylline that is insufficient to inhibit DNA synthesis by itself should still be able to decrease the degradation of cAMP enough to increase the inhibitory effect of a small amount of exogenous cAMP. Table IV contains results pooled from three separate experiments in which treatment of nonconfluent cultures with 0.45 mM theophylline and 0.225 mM cAMP for 24 hr caused a greater inhibition of DNA synthesis than the sum of the effects of either inhibitor alone. This result, which is compatible with the idea that the growth inhibitory action of theophylline is due to its effect on cAMP metabolism, strengthens the conclusion reached on the basis of the data in Table III that the stimulation of cell growth in confluent cultures after refeeding with fresh medium is due to a decrease in cAMP content.

Kinetics of Irreversible Commitment to DNA Synthesis

In this experiment, four sets of cultures grown to confluence in medium containing 10% serum were stimulated with fresh medium. Two sets were subsequently refed at various times after stimulation with fresh medium containing either 0.25 mM db-cAMP or 1.4 mM theophylline. A third set was refed at these same times with contact-inhibited medium. This medium was unable to stimulate DNA synthesis in contact-inhibited cells (Fig. 3), presumably due to a depletion of the serum stimulatory factor as a result of previous incubation on confluent cultures. DNA synthesis for all cultures in these three sets was determined at 19 hr after the original stimulation. The fourth group of stimulated cultures served as controls. Cultures in this set were pulsed at 7, 11, 15, and 19 hr to determine the normal kinetics of synthesis after refeeding.

The results (Fig. 8) are expressed as a percentage of the synthesis in control cultures at 19 hr. The

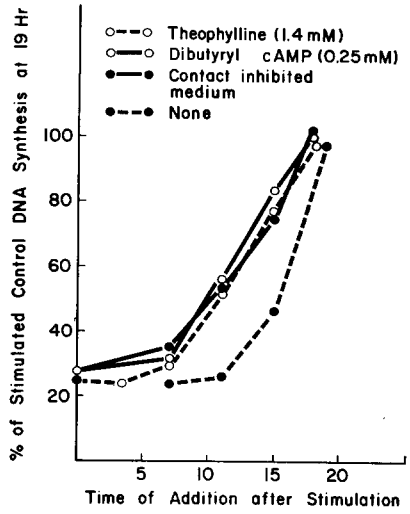

FIGURE 8 Kinetics of irreversible commitment to DNA synthesis. Four groups of confluent cultures were refed with EMEM containing 10% serum. DNA synthesis in one group was followed as a function of time after stimulation (●-----●). The synthesis in this group at 19 hr served as the control value. At the times after stimulation indicated on the abscissa, cultures in the other three groups were refed with contact-inhibited medium or with fresh medium containing either 1.4 mM theophylline or 0.25 mM db-cAMP. DNA synthesis for all cultures in these three groups was determined 19 hr after the initial stimulation and plotted as a function of the time at which one of the inhibitory media was added to the stimulated cultures. All levels of synthesis are expressed as a percentage of the synthesis in the control group at 19 hr. Each point is the average of two determinations.

fact that the upper three curves are almost identical indicates that at the same time cultures became refractory to the inhibitory effects of 0.25 mM db-cAMP or 1.4 mM theophylline on the stimulation of DNA synthesis observed at 19 hr; they also lost their requirement for the macromolecular stimulatory factor present in fresh medium. In terms of irreversible commitment to DNA synthesis, addition of 0.25 mM db-cAMP or 1.4 mM theophylline to fresh medium yielded the same kinetics as removing the growth stimulatory factor from that medium. The direct relationship between the necessity of fresh serum for stimulation and the sensitivity of that stimulation to inhibition by cAMP is a further indication that the serum factor in fresh medium probably stimulates growth and subsequent DNA synthesis in confluent cultures by lowering intracellular cAMP. Also consistent with this hypothesis are the data in Table II which demonstrated that cAMP levels remained low for at least 10 hr after stimulation. During the period which extends up to 4–5 hr before S phase, high levels of cAMP would still be able to inhibit the initiation of DNA synthesis in stimulated cultures (Fig. 8).

DISCUSSION

The results in this paper lend considerable support to the previously suggested idea that cAMP is involved in density-dependent inhibition of cell proliferation (13, 20, 29, 30). Early events in G_1 leading to DNA synthesis in cultures released from contact-inhibition by refeeding were inhibited by theophylline, db-cAMP, and cAMP (Fig. 5). A 30-hr pretreatment of nonconfluent cells with 0.20 mM db-cAMP or 1.4 mM theophylline mimicked the effects of cell-to-cell contact by partially synchronizing their growth in the G_1 phase of the cell cycle (Fig. 7). In addition, confluent cultures refed with fresh medium lost their serum factor requirement for the stimulation of DNA synthesis, which was measured 19 hr after at the refeeding, at same time that they became refractory to the inhibitory effects of 0.25 mM db-cAMP or 1.4 mM theophylline on that stimulation (Fig. 8). Together, these data indicate that cAMP affects DNA synthesis in a manner consistent with the role postulated for it as a physiological regulator of proliferative activity in cultures of human diploid fibroblasts.

In addition, radioimmunoassay measurements demonstrated that cAMP levels in cultures grown to confluence in EMEM containing 1.3% fetal calf serum decreased during the first hour after stimulation with medium containing 10% serum and remained low at 2, 5, and 10 hr (Table II). Moreover, theophylline completely inhibited the 36% decrease in cAMP levels observed 2 hr after stimulation of cultures grown to confluence in 10% serum (Table III) at the same concentration which was required to inhibit completely the stimulation of DNA synthesis in such cultures (Fig. 4). Since the major growth inhibitory effect of theophylline is possibly due to its effect on cAMP metabolism (Table IV), these data suggest that stimulation of cell growth in contact-inhibited cultures by fresh medium requires a decrease in intracellular cAMP.

This conclusion can be made only if theophylline has no action on cell growth other than inhibition of the cAMP phosphodiesterase. Effects independent of its action on cAMP metabolism would tend to weaken the conclusion. Indeed, anomalous effects of theophylline on the production of steroid hormones by adrenocorticotropic hormone (ACTH)-stimulated adrenal glands have been reported (12) and are thought to involve an inhibition of protein synthesis. However, concentrations of theophylline which inhibited cell proliferation in the present study did so without inhibiting over-all protein synthesis (results not shown).

The potentiation of calcium-dependent muscle contraction by phosphodiesterase inhibitors such as caffeine (28) demonstrates their action on calcium uptake by cellular membranes. Since calcium metabolism itself may be important in the control of cell growth, this activity of theophylline might contribute to its inhibitory effect on cell proliferation.

Nonetheless, in spite of its other possible actions, several lines of evidence indicate that theophylline inhibited the growth of the cells used in this study by affecting cAMP metabolism. First, the growth inhibition seen with theophylline occurred at concentrations known to inhibit the phosphodiesterase (2). Moreover, 0.6 mM theophylline inhibited the stimulation of DNA synthesis in confluent cultures after a medium change to a greater extent than 0.6 mM caffeine (data not shown), a result consistent with theophylline's greater inhibitory effect on the cAMP phosphodiesterase (2).

Other data in this paper, as summarized in the first paragraph of the Discussion, indicated that according to the kinetic parameters measured,

theophylline had the same effects on DNA synthesis as cAMP or db-cAMP. This suggests that theophylline might inhibit growth either by acting directly on processes also affected by cAMP or by causing increases in cAMP levels, thereby indirectly effecting inhibition of growth.

However, in another study, Burk (1) showed that the growth of normal BHK cells and RSV-transformed BHK cells was greatly inhibited by 2 mM theophylline while the growth of polyoma-transformed BHK cells was not. Furthermore, he found that normal BHK cells and RSV-transformed BHK cells contained more adenyl cyclase activity in their membranes than the polyoma-transformed cells. This correlation between membrane adenyl cyclase activity and sensitivity to inhibition of growth by theophylline is evidence that a certain amount of cAMP production may be required for theophylline to inhibit growth. If this is true, then the similarity between the effects of cAMP and theophylline on DNA synthesis in the present study can be explained more easily on the basis of theophylline's action on cAMP metabolism than on the basis of one of its other possible effects.

Finally, the data in Table IV demonstrated that 0.45 mM theophylline, which by itself caused no inhibition of DNA synthesis in nonconfluent cultures, was able to increase the inhibitory effect of a 24-hr treatment with 0.225 mM cAMP. This also is consistent with the idea that theophylline inhibits proliferative activity in fibroblast cultures by affecting the activity of the cAMP phosphodiesterase. Thus, the conclusion reached in this paper that a certain level of cAMP is necessary in order for cell growth to be arrested in confluent cultures of normal fibroblasts appears to be valid. Of course, this conclusion could still be weakened by any study which demonstrated an effect of theophylline on cell growth which was independent of its effect on cAMP metabolism.

If one considers the stimulation of cell growth in confluent cultures after a fresh medium change as being the reverse of the process by which the growth of cells is arrested upon reaching confluence, one would be able to postulate that normal fibroblasts stop growing at confluence because of density-dependent increases in cAMP content. Otten et al. (20) have shown that nonconfluent cultures of normal and virus-transformed 3T3 cells contain low levels of cAMP and that cAMP levels increase at confluence in cultures of normal cells but not in cultures of transformed fibroblasts. If these results are generally valid, then the data presented in this paper are strong evidence that contact-inhibition is mediated by increases in cAMP induced at culture confluence.

However, Sheppard (30) has recently shown that cAMP levels in normal 3T3 cells are always higher than those in transformed cells, even in nonconfluent cultures. Furthermore, he has also shown that cAMP levels in normal fibroblasts do not increase at confluence. This is obviously contradictory to the data of Otten et al. and leads to a different interpretation concerning the involvement of cAMP in contact-inhibition. If Sheppard's data are correct, then cAMP may just play a permissive role in contact-inhibition. That is, a high level of cAMP may only be required to alter the cell in such a way that it can react to cell-to-cell interaction with a decreased rate of growth. The restoration of normal surface membrane properties to polyoma-transformed 3T3 cells by db-cAMP is consistent with this (29). Furthermore, according to this scheme, decreases in cAMP would be able to stimulate cell growth in contact-inhibited cultures.

On the other hand, cAMP might be directly inhibitory to growth, but cell-to-cell interaction may be necessary in order for a certain level of cAMP to affect growth. In other words, even if nonconfluent cells contain just as much cAMP as confluent cells, cell-to-cell interaction between normal fibroblasts may sensitize the confluent cells to the growth-inhibitory effects of cAMP. If this is true, then although nonconfluent cells may be less sensitive to inhibition of growth by any given level of cAMP than confluent cells, there should be a level of cAMP which does affect the growth of nonconfluent cells. The fact that exogenous cAMP can arrest the growth of cells in the G_1 phase (Fig. 7) is therefore consistent with the idea that cAMP can inhibit cell growth directly. In any event, the data presented in this paper strongly support an inhibitory role for cAMP in the regulation of cell growth in fibroblast cultures.

The exact mechanism for the inhibitory action of cAMP on cell growth is presently unknown. A number of studies indicate that cAMP probably affects a great many physiological processes within the cell. In bacteria, for instance, cAMP alters the synthesis of inducible enzymes at both the transcriptional (35) and translational (21) levels. Within mammalian cells, cAMP has been shown

to activate protein kinases, enzymes that phosphorylate proteins. The protein phosphorylated by these kinases include basic proteins such as histones (14), enzymes such as phosphorylase (23), and other noncellular proteins such as casein (24).

Phosphorylation of f1 histones by cAMP-activated kinases, demonstrated both in vivo and in vitro (14), suggests that cAMP might alter cell growth processes at the transcriptional level by affecting the types and amounts of RNA produced within the cell. Recent studies on contact inhibition demonstrate control of 28s and 18s rRNA metabolism at the levels of both synthesis and degradation (6). Perhaps cAMP-dependent histone phosphorylations result in a decline in the relative template activity of chromatin specific for rRNA while increasing template activities of chromatin not related to cell growth. Moreover, activation of specific nucleases by phosphorylation might account for the increased degradation of rRNA's in contact-inhibited cells.

Other mechanisms for control of transcriptional events related to growth are also possible. Acidic nuclear proteins have been shown to restore the histone-inhibited DNA-dependent RNA synthetic activity of chromatin isolated from uterine tissue (33) and are generally thought to be regulators of transcriptional events in mammalian cells. An increased synthesis of such proteins has been demonstrated within 2 hr after stimulation of confluent WI-38 fibroblast monolayers with fresh medium (25). This correlates well with the increased RNA synthesis observed in these stimulated cultures (7). Hence, studies of the effects of cAMP on the synthesis of acidic nuclear proteins could offer some insight into how cAMP inhibits cell proliferation.

At the translational level, kinases activated by cAMP have been implicated in the phosphorylation of ribosomal subunits (5). These phosphorylations might explain the decreased probability for the initiation of protein synthesis seen in confluent cultures (31). Perhaps phosphorylated ribosomes are altered so that they no longer translate the specific messengers programming cell division.

Phosphorylation of neurotubular proteins has also been found to be stimulated by cAMP (10). Since microtubular proteins may be involved in cell spreading and motility (3), in the formation and maintenance of cell processes (9), and in intracellular organelle movement (8), alterations in these proteins caused by phosphorylation might also interfere with critical functions necessary for growth. Further investigation is needed to determine how cAMP inhibits cellular proliferation.

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