Title
The role of cyclic nucleotides in the regulation of cell growth

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THE ROLE OF CYCLIC NUCLEOTIDES IN THE REGULATION OF CELL GROWTH

by

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Introduction

In many organs of the mature mammal only a few types of cells undergo division under normal circumstances. These organs, such as skin and intestinal epithelium, continuously renew themselves. Contained within these tissues are subpopulations of cells which undergo continuous proliferation. The growth of these cells is controlled by intracellular regulators. The mechanism of control has not been defined, but cyclic nucleotides have been implicated in the regulation of proliferation. Examples will be discussed in the appropriate section.

Cyclic Nucleotides as Intracellular Regulators

A. Endogenous Levels

1. Cyclic Nucleotides and the Cell Cycle

Until recently, most research on cyclic nucleotides has focused on the role of cyclic 3',5'-adenosine monophosphate (cAMP), but information is beginning to emerge on the role of cyclic 3',5'-guanosine monophosphate (cGMP). I will introduce research on these two cyclic nucleotides separately.

Under basal physiological conditions cAMP is continually being synthesized from ATP by adenyl cyclase. In 1960, Sutherland and Rall demonstrated that some hormonal responses in liver cells were mediated through the production of cyclic AMP by adenyl cyclase. This is an example of the second messenger concept, in which adenyl cyclase and its regulatory elements are presumed to translate changes in the

external environment into changes in intracellular cAMP levels. This is accomplished when hormones, or other regulatory substances, bind to specific receptors on the cell membrane, which in turn signal increased production of cAMP via the membrane-bound adenyl cyclase. In this concept, the hormone or drug is the primary or first messenger, and the cAMP is the second messenger. The level of cyclic nucleotide in the cell is a result of a balance between two opposing reactions: degradation due to a phosphodiesterase and synthesis by cyclases.

Does cAMP play a major role in the regulation of growth? If cAMP does play a role, then we would expect its level to vary with the stages of growth. A change in level would signal the onset of a new phase in the growth cycle. When WI-38 human diploid fibroblasts (1), human skin fibroblasts (2), normal rat kidney fibroblasts (3), mouse fibroblasts, monkey fibroblasts, and hamster fibroblasts (4) reach confluence their cAMP levels showed sharp elevations concomittant with the cessation of growth. Sheppard (5) has reported that density-dependent cessation of growth is not correlated with elevated cAMP levels, but an increase in cAMP levels does occur earlier in growth when the growing cells first contact one another. Other researchers (6,7) have reported that cAMP levels may actually decrease at the time of contact inhibition. It also has been demonstrated that 3T3 cells show no increase in cAMP levels if the medium is changed frequently.

The addition of serum to quiescent cultures of fibroblasts results in renewed growth and is accompanied by a fall in cAMP level (6,8,9,10, 11). Sheppard (5) has shown that serum causes a rapid, transitory decrease in cAMP levels in 3T3 fibroblasts. Serum addition to quiescent cultures of Balb/c 3T3 is accompanied by a transient decrease in cAMP

levels, as well as a transient increase in cGMP levels, with DNA synthesis following 20 hours later (12).

In culture, the condition of the medium determines both the levels of cAMP and the proliferative state of the cells. The inclusion of dibutyryl cAMP in fresh serum blocked a renewed round of cell division of confluent cells (13). Incubation of confluent WI-38 cells in 8-methylthioadenosine 3',5' cyclic monophosphate or agents which cause a rise in cAMP (e.g. prostaglandin E_1 in the presence of aminophyline) has also been reported to block serum stimulation of renewed growth. This evidence supports the theory that cAMP may act as a second messenger, meaning that it translated external conditions into the language of proliferation or non-proliferation. This theory is supported by the studies cited which correlate changes in cAMP levels with changes in the nutrient media. More studies will be discussed in the next section.

Some cell types are stimulated to grow by high intracellular levels of cAMP, including thymic lymphocytes (14), hematopoietic stem cells (13), salivary acinar cells (16), and adrenocortical cells (17).

In psoriatic lesions, reduced levels of cAMP and increased levels of cGMP correlate with a state of cell proliferation, and agents which increase the cAMP level inhibit epidermal mitosis (18).

In summary, we have seen that in most cases a lower intracellular level of cAMP correlates with growth, while higher levels correlate most often with reduced proliferation. However, we are still faced with those cell types which exhibit a pattern opposite to this; i.e. high levels of cAMP correlate with a growth phase. Cyclic AMP still seems to act as a mediator in cells exhibiting this pattern, but the reaction of the cell to the levels of cAMP is different from the majority of cells studied.

2. Mitotic Agents and Cyclic Nucleotides

If cAMP fulfills the role of second messenger in the regulation of growth, then mitotic agents may alter the basal levels of the nucleotide, or alter the responsiveness of the cell to cAMP.

For example, insulin treatment leads to a transient lowering of cAMP levels, accompanied by DNA synthesis and cell division (5,13,19). In 3T3 cells, insulin induces growth and lowers the activity of adenyl cyclase, thus decreasing the levels of cAMP. A small increase in cGMP also occurs (20).

It has been reported that proteases—trypsin, chymotrypsin, ficin, papain, and subtilisin—caused a rapid, temporary decrease in cAMP levels of confluent 3T3 cells, which was followed by cell division (5,13,21). The inclusion of N^6,2'O-dibutyryl cAMP during the 10-minute protease treatment prevented renewed DNA synthesis in confluent 3T3 cells (21).

The bulk of the evidence supports the theory that cAMP may act as a second messenger between external stimulatory agents and the controlling mechanism of the cells. In only one case was the change in cyclic nucleotide level traced to changes in adenyl cyclase activity. In most cases phosphodiesterases play the dominant role in the change in cAMP levels.

B. Exogenous cAMP and cGMP

In some cell types including both normal and transformed fibroblasts, adding cAMP or dibutyryl cAMP to the media results in suppression of growth (22). Cells are usually arrested in the G_1 phase. In L-929 fibroblasts, dibutyryl cAMP inhibits cell division concommitant

with the inhibition of the incorporation of ^{3}H-thumydine into DNA (23). Added dibutyryl cAMP or phosphodiesterase inhibitors slowed the growth rate of myoblasts and delayed the fusion of myoblasts to form myotubules (which occurs when these cells reach confluency) (24).

As previously mentioned, some cell lines show high cAMP levels during growth, and in some cell lines cAMP may stimulate growth. Thymocyte proliferation is stimulated by cAMP in serum-free media as long as calcium is present (25). Primary and secondary cultures of chick embryo fibroblasts were stimulated by cAMP, monobutyryl cAMP, dibutyryl cAMP, adenosine, ATP, cGMP, and dibutyryl cGMP (26). Adenosine and ATP are known to raise cAMP levels. It also has been reported that embryonic liver and kidney cells are stimulated by dibutyryl cAMP (27).

Some cells do not respond to exogenously added cAMP. Such cells include sea urchin eggs (28) and HTC heptoma cells (29,30).

Biological Role of cGMP

Cyclic GMP has been detected in all phyla of the animal kingdom, and is present in a wide variety of tissues. Levels in different tissues may vary from 10^{-8} to 10^{-6} moles/kg (wet weight). The significance of these variations is not understood, and although cGMP has been implicated in several metabolic processes, its metabolic role remains unclear. Clues to the biological role of cGMP can be found in experiments where a change in steady-state cGMP levels correlate with a functional change in the cell brought about by outside agents. Hormones which are associated with increased cAMP levels, such as epinephrin, glucagon, adrenocorticotrophic hormone, parathyroid hormone, or anti-diuretic

hormone do not affect cGMP levels (31,32,33). Hardman, Davis and Sutherland (33,34) demonstrated that the levels of the two cyclic nucleotides may be controlled by different biological factors when they reported that removal of the pituitary gland produced selective lowering of urinary cGMP excretion with little influence on cAMP excretion.

To date emphasis has been placed on the hypothesis that cAMP and cGMP play a dual regulatory role with the two cyclic nucleotides promoting opposing biological events. An initial observation along this line was that acetylcholine-induced depression of cardiac contractivity is associated with a relatively rapid accumulation of tissue cGMP. This action is opposite to that promoted by epinephrin, which elevated myocardial cAMP. Since this observation was made, cholinergic action in other tissues has been correlated with an increase in cGMP levels.

Other agents which have cAMP-opposing characteristics have been examined for their effects on tissue cGMP accumulation. Kaminsky et al. (35) demonstrated that human plasma cGMP levels were elevated as a result of alpha-andrenergic stimulation and that beta-andrenergic treatment resulted in increased cAMP concentrations.

A few studies have linked cGMP with endocrine or neuroendocrine function. For example, adrenalectomy caused a lowering of urinary excretion of cGMP, which could be reversed by glucocorticoid administration (33,34). Adrenalectomy also produced an increased concentration of cGMP in lung and renal tissue (36). Hypoparathyroidectomy produced lowered urinary excretion of cGMP which was reversed by injections of thyroxine. Other studies also have associated glucocorticoid levels

with changes in cGMP levels. No clear relationship regarding function has yet been established.

cGMP Levels and Cell Growth

Mitogenic agents have been known to stimulate growth and raise cGMP levels. Concanavalin A and phytohemaglutinin increase lymphocyte proliferation and cGMP levels without altering cAMP levels (37). Serum stimulated growth of 3T3 cells occurred concurrently with a rise in cGMP levels and a fall in cAMP levels (12). Insulin increased DNA synthesis and a rise in cGMP levels.

Cyclic GMP has been reported to play a part in other regulatory roles within the cell. In 3T3 cells, cGMP reversed the effects of PGE_1, which depressed protein and RNA synthesis (38).

To date not much research has been done on added cGMP and cell growth. Cyclic GMP, 8-bromo-cGMP, and $N^6,2^8O$-dibutyryl cyclic GMP have been shown to increase ^3H-thymidine and ^3H-uridine uptake into nucleic acids of Balb/c mouse spleen lymphocytes (41). It has been reported that cGMP or its butyrated analogs, along with hydrocortisone and bovine serum albumin, stimulate thymidine incorporation into about 10% of the nuclei of quiescent Balb/c 3T3 fibroblasts (21).

Cyclic Nucleotides and Transformed Fibroblasts

It is beyond the scope of this paper to discuss fully the work done on cyclic nucleotides and neoplasia, but this area does deserve mention.

It has been postulated that decreased cAMP levels may play a role in the lack of growth inhibition in transformed cells (5). Transformed

3T3 fibroblasts have basal cAMP levels about one-half the level of their normal counterparts; however, fluctuations in the intracellular cycle of transformed and normal 3T3 fibroblasts are very similar (5).

In contrast to the above data, it has been reported that cAMP levels were greatly elevated in a variety of Morris hepatoma explants, in comparison to normal rat liver, and no correlation between cAMP level and growth rate could be made. Transplanted rat adrenocortical carcinoma 494 was found to contain about twice as much cAMP as normal rat adrenal gland (44).

Purpose of My Experiments

My experiments were undertaken to explore further the role of cGMP in the control of growth. The specific focus was to determine whether or not added cGMP stimulates growth. I also explored the effects of varying culture conditions.

As will be seen, I demonstrated that in certain cells, under specified conditions, cGMP does indeed stimulate growth.

Materials and Methods

Media was purchased from Grand Island Biological. Either Dulbecco's Modified Minimal Essential Media or RPMI were used. The Dulbecco's Media was used in two forms: 1) Low Glucose (1000mg/ml); or 2) High Glucose (4500mg/ml). Both media were supplemented with 10% Fetal Bovine Serum except where otherwise stated, and Hepes-Tes-Bes buffer [Hepes is (N-2-Hydroxyethyl) piperazine-N^1-2-ethane sulforic acid; Bes is (N,N-bis 2 Hydroxymethyl-2-Aminoethane Sulforic acid; Tres is (N-tris Hydroxymethyl methyl)-2-aminoethane sulforic acid], as described by Eagles (45), was used instead of CO_2-Bicarbonate buffer to maintain the proper degree of acidity.

Cells were maintained in 8 oz. plastic flasks, tightly stoppered with rubber stoppers, at 37° C. Cells were grown in disposable glass tubes, tightly stoppered with rubber stoppers, during the course of the experiment. Twenty-four hours after tubes were planted with cells, the appropriate media was supplemented with 20 microliters of media or 8Br-cGMP (obtained from Sigma Chemical Company, St. Louis) dissolved in media, or media alone. After the indicated time, the cells were lysed with a 0.01M citric acid -5% cetavalon solution, and the nuclei counted on a particle counter (Coulter, model B). Each experimental value was measured using 5-6 tubes and the cells in each tube were counted twice.

L-929 Cells

These cells are mouse fibroblast and were obtained from the National Center for Tissue Culture.

Results.—Refer to Table I.

The first experiment was carried out with cells growing in RPMI-HTB buffer. A small inoculum of cells was used because a larger, initial inoculum caused sheets of cells to peel off the wall before 72 hours had elapsed. Two concentrations of cGMP were used, 10^{-6}M and 10^{-3}M, and cell counts were taken at 48 hours and 72 hours after the addition of 8Br-cGMP. There is a very large stimulation in growth in the cells growing in 10^{-3}M 8Br-cGMP, and to a lesser extent in cells growing in 10^{-6}M 8Br-cGMP. By 72 hours the gap between the experimentals and the controls has narrowed. This is partly due to the fact that the control cells have entered the log phase of the growth curve, while the experimentals are coming out of this phase and thus their growth rate is leveling.

In the second experiment, cells were grown in DEM-high glucose. There was significant stimulation in growth by 10^{-8}M 8Br-cGMP, 10^{-6}M 8Br-cGMP, and 10^{-3}M 8Br-cGMP by 24 hours, but this effect diminished by 48 hours in a pattern similar to the first experiment.

The experiment was tried using DEM-low glucose medium, but under these conditions there was no effect on growth rate.

Summary.—The overall effect of 8Br-cGMP at all concentrations tested was to shift the growth curve to the left, meaning that the exponential growth phase was reached sooner, but the plateau corresponded to the same cell density in both the controls and the experimentals. It did not result in a sustained increase in the number of cells. The reasons for this are unclear.

L-929 TABLE I

Culture Conditions	48 HOURS				72 HOURS			
	Control	Experimental 10^{-6}M cGMP	% Stim	P**	Control	Experimental 10^{-6}M cGMP	% Stim	P**
Experiment 1: RPMI - 10%FBS	3,800 ± 400	6,000 ± 1270	56%	P .025	6,300 ± 290	7,500 ± 290	19%	P <.01
	Control	Experimental 10^{-3}McGMP			Control	Experimental 10^{-3}McGMP		
	3,800 ± 400	7,700 ± 1200	101%	P .001	6,300 ± 289	9.000 ± 1,1900	43%	P <.01

Experiment 2: DEM-HG* - 10%FBS	24 HOURS				48 HOURS			
	Control	Experimental 10^{-8}M cGMP	% Stim	P	Control	Experimental 10^{-8}M cGMP	% Stim	P
	4,700 ± 230	6,830 ± 430	45%	P .005	6,455 ± 1380	6,710 ± 688	-	Not significant
	Control	Experimental 10^{-6}McGMP			Control	Experimental 10^{-6}McGMP		
	4,700 ± 230	6,760 ± 1,730	44%	P .05	6,455 ± 1380	6,710 ± 688	-	Not significant
	Control	Experimental 10^{-4}McGMP			Control	Experimental 10^{-4}McGMP		
	4,700 ± 231	6,960 ± 1,400	48%	P .05	6,455 ± 1380	8,040 ± 2,443	-	Not significant

*Dulbecco's modified minimal essential medium—high glucose.
** - Calculated by Student's method.

L Cells

These cells are mouse fibrosarcoma and were obtained from the National Center for Tissue Culture.

Results.—Refer to Table II.

In the first experiment we compared the effects of media containing 10% FBS and 10% dialyzed plasma. Since 8Br-cGMP is very stable, I assumed it would be equally as stable in serum and plasma. The medium was DEM-low glucose. Under the conditions of 10% FBS, 10^{-3}M 8Br-cGMP produces a significant depression in growth by 72 hours. In the cells in which serum is replaced by dialyzed plasma, once again significant depression in growth is evident by 48 hours, for both cells growing in 10^{-6}M 8Br-cGMP and 10^{-3}M 8Br-cGMP, and depression is enhanced by 72 hours.

The second experiment confirms growth depression in DEM-low glucose media with 10% serum for cells in the presence of 10^{-3}M 8Br-cGMP.

The third experiment examines the effect of switching to DEM-high glucose media. We also compared the effects of 3% serum with 10% serum. Growth depression was not observed when cells were grown in 3% serum, but once again 10^{-3}M 8Br-cGMP produced growth depression by 72 hours for cells growing in 10% serum.

Summary.—In all experiments in which 10% serum was present, 10^{-3}M 8Br-cGMP inhibited growth. This was not affected by the glucose concentration of the media. However, 3% serum did not support this effect. The presence of 10% dialyzed plasma also supported growth inhibition by cGMP.

L CELL TABLE II

CULTURE CONDITIONS	48 HOURS				72 HOURS			
Experiment 1:	Control	Experimental	% Depression	P*	Control	Experimental	% Depression	P*
1a DEM-LG[1] 10%FBS					19,200 ± 2,600	10,600 ± 2,800	44.8%	P<.01
	Control	10^{-6} McGMP			Control	10^{-6} MGMP		
1b DEM-LG[1] 10% dialyzed plasma	19,100 ± 1,660	12,200 ± 1,390	36.1%	P .005	38,800 ± 2,880	18,400 ± 650	52.54%	P<.005
	Control	10^{-3} McGMP			Control	10^{-3} McGMP		
	19,100 ± 1,660	13,000 ± 908	31.9%	P .005	38,800 ± 2,880	16,100 ± 800	58.5%	P<.005
Experiment 2 DEM-LG[1] 10%FBS	Control	10^{-3} McGMP			Control	10^{-3} McGMP		
	22,400 ± 1,690	13,900 ± 1,380	37.9%	P .0005	37,300 ± 1,800	22,500 ± 3,680	39.7%	P<.005
Experiment 3 DEM-HG[2] 3%FBS					Control	10^{-3} McGMP		
					2,700 ± 650	1,900 ± 340	-	Not significant
DEM-HG[2] 10%FBS					Control	10^{-3} McGMP		
					8,000 ± 1,130	5,000 ± 840	37.6%	P<.005

*P - calculated by student's method.

[1]Delbecco's modified minimal essential medium—low glucose.

[2]Delbecco's modified minimal essential medium—high glucose.

NRK Cells

These cells are from rat kidney. They were obtained as a gift from Dr. J. Levy, University of California at San Francisco.

I. Cyclic GMP

The first experiment (see Table III) contrasts cell growth in dialyzed plasma with cell growth in serum. The media used was RPMI. A very pronounced stimulation was observed in the plasma-supported cells by 72 hours for both the 10^{-6}M cGMP and 10^{-4}M cGMP. Stimulation also was observed in the serum-supported cells, but was not as strong as in the cells growing in plasma. The difference between the effects seen in the plasma experiment and the serum experiment reflect both the fact that the control cells grew faster in the serum, while the cells growing in serum and cGMP grew more slowly than those growing in plasma and cGMP.

In the second experiment we repeated the cells-in-plasma experiment only with a lower initial cell plant. Once again RPMI-10% dialized plasma supported significant stimulation by 72 hours for both cells growing in 10^{-6}M cGMP and cells growing in 10^{-3}M cGMP.

In the third experiment I tried the effects of switching to DEM-HG. Serum concentration was tested at 10% FBS, and DEM-HG-10% FBS was compared to DEM-HG-10% FBS-5mM$CaCL_2$. None of these combinations supported cGMP stimulation of cell growth.

Summary.—The combination of RPMI-10% dialyzed plasma consistently supported cGMP stimulation of growth by 72 hours at all concentrations of cGMP tested. However, cGMP mediated stimulation was less consistent when cells were grown in RPMI-10% FBS. These results are puzzling.

NRK - Cyclic Nucleotides: These figures are for 72 hours.

TABLE III

Conditions	Control	Experimental	% Stimulation	P*
Experiment 1				
1a RPMI - 10% plasma	Control	10^{-6} McGMP		
	24,500 ± 5,780	48,800 ± 7,000	99%	P<.0015
	Control	10^{-4} McGMP		
	24,500 ± 5,766	37,700 ± 1,786	51%	P<.005
1b RPMI - 10% serum	Control	10^{-6} McGMP		
	25,200 ± 5,740	34,300 ± 2,630	36%	P<.01
	Control	10^{-4} McGMP		
	25,200 ± 5,740	32,900 ± 600	31%	P<.025
Experiment 2				
RPMI - 10% plasma	Control	10^{-6} McGMP		
	6,400 ± 680	9,800 ± 1,120	53%	P<.005
	Control	10^{-4} MCGMP		
	6,400 ± 680	9,700 ± 350	51%	P<.0005

*P - Calculated by Student's Method.

The lack of stimulation in cells growing in serum may be due to the interference of cyclic nucleotides already present in the serum. Even more surprising is that DEM-HG did not support cGMP stimulation regardless of the serum concentration.

II. Effects of Other Nucleotides and Nucleosides on NRK Cells:

First Experiment

Method.—This experiment was carried out in the same manner (see Table III) as the experiments using cGMP, except that in these experiments cells were grown in 10^{-4}M guanosine, 10^{-4}M GMP, 10^{-4}M 8Br-GMP, and 10^{-4}M AMP. The medium was DEM-HG-10% FBS.

Results.—None of these substances had any effect on growth within the 72 hours tested.

Second Experiment

Method.—This was essentially a repetition of the first experiment, but the following concentrations of substances were used in addition to those used above: 10^{-6}M guanosine, 10^{-6}M GMP, 10^{-6}M 8Br-GMP, and 10^{-6}M AMP.

Results.—Once again, none of these substances altered the growth pattern during the 72 hours tested.

III. Effects of Cyclic Nucleotides in Long-term Experiments on NRK Cells:

First Experiment (See Table IV)

Method.—In the first experiment, the 8Br-cGMP was not added until 96 hours after planting. The addition of 8Br-cGMP marked the beginning of the experiment, and time was measured from this point. The medium was not changed during the entire course of this experiment, yet it did

NRK Long Term Experiments TABLE IV

Conditions	Control	Experimental	108 Hours % Depression	p**
Experiment 1				
1a DEM-HG* 3%FBS	Control	10^{-6} McGMP		
	23,400 ± 2,150	11,700 ± 3,460	50%	p<.001
	Control	10^{-4} McGMP		
	23,400 ± 2,150	9,600 ± 600	57%	p<.0005
1b DEM-HG* 10%FBS	Control	10^{-6} McGMP		
	12,400 ± 570	7,900 ± 2,440	36.5%	p<.01
	Control	10^{-4} McGMP		
	12,400 ± 570	6,000 ± 1,000	51.5%	p<.0005
Experiment 2				
2a DEM-HG* 3%FBS	Control	10^{-6} McGMP		
	10,500 ± 2,950	9,500 ± 1,600	no effect	
	Control	10^{-4} McGMP		
	10,500 ± 2,950	4,200 ± 700	60.5%	p<.0005
2b DEM-HG* 10%FBS	Control	10^{-6} McGMP		
	8,600 ± 1,940	8,900 ± 2,500	no effect	
	Control	10^{-4} McGMP		
	8,600 ± 1,940	10,000 ± 1,750	no effect	

*Dulbecco's modified minimal essential medium—high glucose.
**P ¬ Calculated by Student's method.

not become acidic. This experiment was carried out in both DEM-HG-3% FBS and DEM-HG-10% FBS.

Results.—This experiment demonstrates consistent and marked depression of cells growing in 8Br-cGMP for all concentrations tested by 108 hours. Serum concentration did not affect the cyclic nucleotide mediated depression, but it is interesting to note that the control cells grew better in media containing 3% FBS than in media containing 10% FBS.

Second Experiment (see Table IV)

Method.—This time cGMP was added at 24 hours after planting, as usual. This was assigned the value of time = 0. Cell counts were taken at 72 hours, 96 hours, and 108 hours. The media were not changed during the course of the experiment. The media used were DEM-HG-3% FBS and DEM-HG-10% FBS.

Results.—In the cells growing in media containing 3% FBS, there was a marked depression in growth of cells subjected to 10^{-4} 8Br-cGMP by 108 hours, yet the less concentrated 10^{-6} 8Br-cGMP failed to mediate a significant depression in growth.

In the cells growing in media containing 10% FBS, depression of growth appeared at 96 hours for cells growing in both 10^{-6} M 8Br-cGMP and 10^{-4} M 8Br-cGMP. However, by 108 hours there was no difference between the experimentals and the controls.

Other Cell Types

Hela Cells

These cells were obtained from the National Center for Tissue Culture.

See Table V.

Hela cells were tested in RPMI containing 10% dialyzed plasma versus the same medium with 10% serum. Under the conditions of plasma, 10^{-4}M 8Br-cGMP produced significant growth depression by 72 hours, while media supplemented with 10% serum supported growth stimulation for 10^{-4}M 8Br-cGMP. This experiment indicates that the culture conditions are very important to the effects observed by cyclic nucleotides.

Balb c/3T3

Cells were obtained from the Cell Culture Laboratory, Naval Biomedical Research Laboratory, School of Public Health, University of California at Berkeley.

Cyclic GMP did not produce any significant results in this cell line. Experiments were carried out in DEM-high glucose, with both 3% serum and 10% serum.

AP 510

Cells were obtained from the Cell Culture Laboratory, Naval Biomedical Research Laboratory, School of Public Health, University of California at Berkeley.

As with the 3T3 cells, 8BR-cGMP did not produce significant results in this cell line. Experiments were carried out using DEM-high glucose.

Hela-Cyclic Nucleotide

TABLE V

Conditions	Control	Experimental	Effect	P
1. RMPI - 10% plasma	Control	10^{-4} McGMP		
	22,800 ± 5,280	15,200 ± 1,310	33% depression	P<.01
	Control	10^{-4} McGMP		
RPMI - 10%FBS	24,600 ± 3,180	30,400 ± 240	23% stimulation	P<.05

P - Calculated by Student's Method.

Phosphodiesterase Inhibitor

Refer to Table VI.

Cyclic AMP phosphodiesterase is an enzyme which degrades cAMP to AMP. The effect of a phosphodiesterase inhibitor is to block the action of phosphodiesterase and thus raise intracellular concentration of cAMP. Many agents which affect the level of cAMP also change the level of cGMP. The phosphodiesterase inhibitor acts mainly on the cAMP phosphodiesterases so that it will raise intracellular cAMP without greatly affecting the cGMP levels.

Several agents are known to inhibit cyclic AMP phosphodiesterase activity. Among them are theophyline, papaverine, and RO20-1742 [D,L-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidmone], which will be abbreviated in this paper as RO. Although the mechanism of action of RO is not known, it appears to be quite stable. In one study it was found to produce a 50% inhibition at a concentration of 2.5×10^{-3}M (39).

RO has been reported to increase the response of neuroblastoma cells to adenosine, although basal cAMP levels were unaffected in one neuroblastoma clone and slightly raised in another (40).

Materials and Method.—RO was obtained as a gift from Dr. Sheppard of Hoffmann-La Roche, Inc., Nutley, New Jersey. The procedure in this experiment was identical to that used in the cyclic nucleotide experiments, except that RO was added instead of a cyclic nucleotide.

Results.—In the first experiment, I compared the effects of two different concentrations of RO. While 10^{-6}M RO did not mediate an effect on growth, 10^{-4}M RO inhibited growth completely. Under these conditions, the number of cells present after 72 hours is equivalent to

TABLE VI

Hela Cells

Conditions	Control	Experimental	% Depression	P*	Control	Experimental	% Inhibition	P*
RPMI - 10%FBS	Control	10^{-4} M RO			Control	10^{-4} RO		
	10,340 ± 790	5,860 ± 1,047	43%	P<.0005	21,200 ± 1340	5,350 ± 360	74%	P<.0005
RPMI - 10%FBS	Control	10^{-4}MRO			Control	10^{-4}M RO		
	43,100 ± 1610	15,900 ± 1380	63%	P<.0005	56.732 ± 8,863	9,000 ± 1,630	84%	P<.0005
8Br-cAMP	Control	10^{-4}M 8Br-cAMP			Control	10^{-4}M 8Br-cAMP		
	43,100 ± 1600	22,100 ± 2,900	48%	P<.01	56,732 ± 8,863	25,200 ± 5,340	50%	P<.01

*P - Calculated by Student's method.

the number planted.

In the second experiment I compared the effects of 8Br-cAMP and 10^{-4}M RO in inhibiting growth. Once again RO completely halted growth by 48 hours. Growth was also depressed by 10^{-4}M 8Br-cAMP by 48 hours and extending into 72 hours. It can be seen from the table that RO is a more effective inhibitor of growth than exogenously added 8Br-cAMP.

Phosphodiesterase Inhibitor and Intracellular Levels of Cyclic Nucleotides

In the experiments on Hela cells, it was shown that phosphodiesterase inhibitor was more effective in slowing growth of cells than 8Br-cAMP. In order to investigate this result further, I decided to measure the levels of cAMP and cGMP in Hela cells growing in the presence of RO and Hela cells under normal culture conditions.

Materials and Method.—Hela cells were cultured in DEM-HG 10% FBS for 24 hours after initial planting. At this time, the media was poured off and fresh media was added to the control bottles, and media containing 10^{-4}M RO was added to the experimental bottles. The cAMP and cGMP levels were measured at 4 and at 24 hours by radioimmune assay. The assay procedure was that of Harper and Brooker (41).

Results.—This experiment was repeated twice. At neither 4 hours nor at 24 hours were the cAMP levels significantly higher in the phosphodiesterase inhibitor-treated cells. This indicates that RO does not halt growth via an elevation of cAMP in Hela cells. However, this does not indicate that cAMP could not play a role in the control of growth under normal conditions. Similarly, cGMP levels were not significantly different in the control and experimental cells.

Conclusion

One unknown in my results is the answer to this question: How does the effect of added 8Br-cGMP compare with cGMP? Insight into this question can be gained from the work of others on cyclic nucleotide analogs.

One way to compare the effects of 8Br-cGMP with cGMP is to examine their abilities to activate the enzymes concerned with cyclic nucleotide metabolism. It has been postulated that cGMP may work through the activation of a cGMP-dependent protein kinase. When both of these compounds were tested with lobster cGMP-dependent protein kinase, 8Br-cGMP was more active than cGMP (46,47). Also, the 8BR-cGMP retained its specificity, being a very poor activator of cAMP-dependent protein kinase. As mentioned previously, phosphodiesterase is the enzyme responsible for the hydrolysis of cGMP. When tested with a phosphodiesterase preparation from rabbit kidney, cGMP is a significant substrate, but 8Br-cGMP is not hydrolyzed by phosphodiesterase (46) and is not a significant competitive inhibition of cGMP. This effect was observed previously with enzyme preparations from bovine heart and rabbit lung (47). Because of 8Br-cGMP's greater potency in activating a cGMP-dependent protein kinase and its resistance to hydrolysis, we can expect 8BR-cGMP to be a more potent agonist of cGMP function than cGMP itself.

The one remaining question is whether or not the 8BR-cGMP is able to enter the cells. The activity of several 8-substituted cAMP derivatives in isolated cells (48) and in tissue preparation (49) suggests that the analogs do penetrate cells. We can look, also, at the significant activity of some of the cGMP derivatives in the histamine-induced

bronchospasm and the passive cutaneous anaphylaxsis systems (47) and take this as an indication that the analogs are entering cells. The 8-bromo derivative also was able to mimic the action of cholinergic agents in lung fragments (50), which contributes to the evidence suggesting that cGMP does penetrate the cell.

Taken together, these results indicate that 8Br-cGMP is a useful tool in studying the action of cGMP.

In order to define a role for cyclic GMP in growth, it is important to demonstrate that the cyclic nucleotide itself can produce a definable effect on a target population of cells. The effects of exogenous cGMP should be consistent with the expression of cellular processes associated with elevated intracellular levels of cGMP. In the introduction, I pointed out studies which indicated that intracellular cGMP was associated with a state of proliferation, and my experiments with exogenous cGMP tended to be consistent with this in some cell lines. Cyclic GMP has been postulated to be a positive intracellular signal involved in the regulation of growth, and cAMP a negative intracellular signal in the growth process.

My experiments examined the effects of altering the cGMP levels. The question I attempted to answer is: Is there a correlation between the growth rate of cells and basal cGMP levels? My experiments demonstrated that several cell types are capable of responding to exogenous cGMP. However, the culture conditions in some cases determined whether the response was marked by increased growth rate or depressed growth rate. For example, in the NRK cells, when cGMP was added within 24 hours after planting, an increased rate of proliferation was observed.

However, when cGMP was added later in the growth cycle, growth depression was observed. Another important factor was the serum concentration and the plasma versus serum effects observed. In some of these experiments, where growth was inhibited by higher concentrations of cGMP, it is possible that cGMP was acting to mimic the effects of cAMP. Glinsman and Hern (42) found that at concentrations of 0.1M to 1mM cGMP was nearly as effective as cyclic AMP in promoting glucose output, glycogenolysis, and phosphorylase activation. These effects occurred without any alteration in cAMP levels. Similarly, the growth of several malignant cell lines in culture was found to be inhibited equally as well by cGMP as by cAMP (43). Cyclic GMP shows a unique concentration-dependent effect to mimic the stimulatory effects of cAMP on proliferation of rat thymocytes (44). Therefore, it is not at all unlikely that in some cell lines at higher concentrations cGMP may mimic the actions of cAMP.

Another possibility exists to account for the responses to cGMP exhibited by different cell lines. It could be that lower cGMP levels signal differentiated functions and that higher cGMP levels correspond to a state of undifferentiation. It is well known that the opposite is true in many cell types capable of responding to cAMP; that is, agents which trigger a change in intracellular cAMP also signal differentiated functions. In the case of cAMP, in some cells a state of differentiation corresponds to growth as in thymocytes. In others, such as neurons, differentiation corresponds to a non-proliferative phase. So it may be that cGMP signals a change in the differentiated state, but that different cell types express that change quite differently in terms of growth.

It is not known whether cGMP acts as a mitogen or whether it accelerates the mitotic process in cells which have already been triggered. If the latter is true, then this could account for the different degrees of growth stimulation produced in different experiments. The amount of growth stimulation observed could depend upon the number of cells in the subpopulation which had already entered the mitotic cycle.

In conclusion, my experiments demonstrated that exogenous cGMP produces a measurable and a repeatable effect on growth rates in several cell types. This supports the hypothesis that cGMP plays a role in the growth mechanism. I have advanced several possible explanations to account for the different effects in different cell types. In order for cGMP to be the ultimate regulator of cell growth, we would expect it to produce similar effects in all cell types. My experiments did not show this, and so I feel that cGMP is not the ultimate regulator of the mitogenic cycle.

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