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Cyclic Nucleotides and Growth Control in Cultured Mouse Cells: Correlation of Changes in Intracellular 3′:5′cGMP Concentration with a Specific Phase of the Cell Cycle

(cAMP/nutrient starvation/G_0 arrest/DNA synthesis)

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ABSTRACT **The commencement of cell growth following serum addition to quiescent cultures of mouse fibroblasts is preceded by transient changes in intracellular concentrations of cAMP and cGMP. By artificial depletion of the culture medium for different nutrients, cell growth can be reversibly arrested in various phases of the cell cycle. Here it is shown that the major cGMP increases are only observed when cultures which are arrested in the G_0 phase are stimulated to grow or when synchronized growing cells pass through the G_1 phase. In addition to its concomitant decrease, cAMP exhibits rhythmic changes during the cell cycle. This suggests that the increase in cGMP could act as a specific signal for movement of cells out of the G_0 or G_1 phase of the cell cycle by activating the pleiotypic and mitogenic program of the cell.**

Nontransformed fibroblasts in tissue culture usually exist in two reversible growth states, a state of rapid proliferation (growing) and a state of relative quiescence (resting) (1, 2). Reinitiation of growth of quiescent fibroblasts can be induced by addition of fresh serum (3, 4). DNA synthesis is initiated 14–16 hr later in a roughly synchronous manner followed by mitosis and cell division (1). Cells thus reversibly arrested are said to exist in the G_0 phase of the cell cycle, as the time from mitosis to DNA synthesis for growing cells (G_1) is considerably shorter (5) and there are also biochemical differences between cells in G_0 and G_1 (6). The 3′:5′ cyclic nucleotides cAMP and cGMP were implicated as intracellular signals in the transition from a resting to a growing state, as the cellular concentrations of cAMP transiently fall (7–10) while those of cGMP rise after brief exposure of quiescent mouse fibroblasts to animal serum or insulin (10, 11, 26). Moreover, exogenous additions of high nonphysiological concentrations (10^{-5}–10^{-3} M) of cAMP or cGMP either prevent the initiation of DNA synthesis by serum (12) or induce substantial increases in DNA synthesis (10) in quiescent cultures, respectively. Growing fibroblasts can also be reversibly arrested by starvation for certain low molecular weight nutrients (13–15). In Swiss mouse 3T3 cells this may lead to either random arrest or to a specific arrest in the G_0 phase of the cell cycle. This phase is probably the same G_0 phase as that assigned to cells arrested by serum limitation in that about 14–16 hr are required to reinitiate DNA synthesis upon restoration of the depleted nutrient (15). We now investigate the intracellular cyclic nucleotide changes, and in particular the changes in cGMP, when quiescent mouse fibroblasts, starved for particular nutrients or arrested by serum depletion, are stimulated to reinitiate growth and proceed through the cell cycle. In view of the close analogy between stringent control in bacteria and the pleiotypic response in mammalian cells (16), control of growth by amino-acid levels in the medium is of particular interest.

Abbreviation: DEM, Dulbecco's modified Eagle's medium.

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MATERIALS AND METHODS

Growth and Handling of Cells were as described previously (10, 17). Cells were grown at 37° in a 10% CO_2 atmosphere in 10 ml of Dulbecco's modified Eagle's medium (DEM) containing 10% calf serum in 9-cm tissue culture dishes unless otherwise stated. *Conditions for arrest of cells by nutrient starvation* were substantially as reported for 3T3-4A (15) and SV3T3 cells (18). Swiss 3T3-4A cells plated at 5×10^5 cells per tissue culture dish were grown for 3 days in DEM containing 10% serum. Medium was removed, the cell monolayers were washed twice with DEM lacking the requisite nutrients, and the medium was then replaced with DEM containing $^1/_{100}$th of the concentration of the requisite nutrient in DEM and 10% dialyzed serum. After a subsequent 3–4 days when the cell population per dish remained constant, the regular concentrations of the depleted nutrients were added back for reinitiation of cell growth. The SV3T3 cells (3T3 cells transformed by simian virus 40) were plated at 4×10^5 cells per dish and grown for 3 days in DEM plus 5% serum. After 3 days, the medium was changed to 5% dialyzed serum and DEM containing $^1/_{200}$th of the normal leucine concentration. After two further days when the cell population remained constant, leucine was restored for the reinitiation of cell growth.

Cell Cycle Analysis was performed as described by Holley and Kiernan (15). Cells from about three or four cultures were removed from the dishes with 0.01% trypsin, washed several times in isotonic buffers, fixed in 20% formaldehyde–isotonic saline solution, and incubated overnight at 4°. The stained cells were analyzed in a Los Alamos design microfluorometer with an argon laser at 488 nm for their DNA content (corresponding to G_1-phase, S-phase, and G_2-phase). The photographs obtained from the oscilloscope were analyzed graphically.

DNA Synthesis. Cell cultures were labeled with 3 μCi/ml of [*methyl*-^{3}H]thymidine at either 3 μM for DNA synthesis or 1 μM for radioautography and processed as described pre-

TABLE 1. *Cell cycle analysis*

	Phase		
	G_1	S	G_2
3T3 growing	57	26	17
3T3 resting	94	0	6
3T3 − phosphate	93	0	7
3T3 − histidine − glutamine	94	0	6
3T3 − leucine	72	10	18
3T3 − methionine	71	12	17

The growth of 3T3-4A cell cultures was arrested by depletion for phosphate, histidine, and glutamine, leucine, or methionine as described in *Methods*. Randomly growing cultures of 3T3-4A (3T3 growing) were also included, while 3T3-4A cultures grown in 10% serum were allowed to become quiescent by serum depletion (resting). The percentage of cells in different phases of the cell cycle is recorded (15).

viously (10, 17). Unless otherwise stated, labeling times were throughout the entire DNA synthetic period in synchronized cultures. The cpm of [^{3}H]thymidine incorporated into DNA or the percentage of cells having radioactively labeled nuclei was recorded. *Cell numbers* per 9-cm dish were recorded in a Coulter Counter.

Estimation of Cyclic Nucleotides. The medium from usually 10 cultures on 9-cm dishes was removed, cold 5% trichloroacetic acid was added to the dishes, and the cell extracts were purified by centrifugation, ether extraction, and chromatography over Dowex columns as described previously (10). [^{3}H]Cyclic nucleotides were added to standardize the recoveries. The cyclic nucleotides were estimated by the radioimmune assays of Steiner *et al.* (19). To check the authenticity of the products assayed, approximately half of each sample was digested with 3′:5′ cyclic nucleotide phosphodiesterase and any nondigested material that cross-reacted with the antiserum directed against cGMP or cAMP was deducted to yield the final values (approximately 0.1 and 0.5 pmol per 10^6 cells for cAMP and cGMP, respectively). Results were expressed in pmol per 10^6 cells or pmol/mg of protein. All points are the average of duplicate experiments which agree to within 15%. *Prostaglandin E1*, a kind gift of Dr. Pike, Upjohn Co., was added to some cultures in a final concentration of 0.1% ethanol (v/v). 0.1% Ethanol addition alone had no effect on the intracellular cyclic nucleotide concentration.

RESULTS

The position of cells in the cell cycle can be accurately measured from their DNA content per cell (20). The results in Table 1 confirmed those of Holley and Kiernan (15) that while leucine and methionine starvation of Swiss 3T3-4A cells caused cessation of growth in a random manner with a large proportion in the S and G_2 phases, starvation for phosphate or a combination of histidine and glutamine resulted in the entire population's becoming arrested in the G_1 phase. The kinetics of changes in intracellular cyclic nucleotide concentrations upon re-addition of histidine and glutamine to cultures originally starved for those amino acids are shown in Table 2. The transient increase (10-fold) in cGMP and decrease in cAMP (2-fold) reached a maximum and minimum, respectively, after 10–20 min and approached "resting cell" concentrations after about 1 hr. These changes were very similar to those observed in confluent and quiescent BALB/c 3T3 (10) or 3T3-4A (unpublished results) fibroblasts upon growth initiation by serum. The 60-min value for cGMP was still significantly higher (4-fold) than the zero time value. Of those nutrients tested, only those whose deprivation arrested the cell population in the G_0 phase of the cell cycle (phosphate or glutamine and histidine) led to increases in cGMP (8- to 16-fold) and large decreases in cAMP (60–70%) within 20 min of their restoration. In contrast, leucine or methionine addition to cultures randomly arrested in the cell cycle by their deprivation caused no detectable increase in cGMP and no large decrease (less than 25%) in cAMP (Table 3). Addition of prostaglandin E1 raises intracellular cAMP concentrations and partially suppresses the induction of DNA synthesis after serum addition to quiescent cultures of 3T3-4A cells (7). Prostaglandin E1 addition also suppresses by about 3-fold the rise in intracellular cGMP and the eventual induction of DNA synthesis in 3T3-4A cells originally starved for histidine and glutamine upon re-addition of the depleted amino acids (Table 2).

TABLE 2. *Kinetic changes in cyclic nucleotide concentrations after addition of histidine and glutamine*

Time (min)	pmol/10^6 cells	
	cGMP	cAMP
0	0.5	4.3
10	5.2	2.4
20	4.6	2.3
20 + prostaglandin E1	1.8	11.8
60	2.0	5.0

After 4 days of incubation in medium deficient in histidine and glutamine (6.5 × 10^5 cells per dish), the regular concentrations of histidine and glutamine were added back and the cultures were isolated for cyclic nucleotide determinations at the stated times. Prostaglandin E1 (10 μg/ml) was also added to some cultures immediately before the amino-acid additions and these cultures were isolated 20 min later. Results are expressed in pmol/10^6 cells. Cell cultures were also radioactively labeled from 8 until 30 hr after the time of additions for DNA synthesis and radioautography. Starved cultures incorporated 21,500 cpm (1% labeled nuclei), amino acid activated cultures, 802,000 cpm (86% labeled nuclei), and activated cultures with prostaglandin E1, 307,000 cpm (50% labeled nuclei) into DNA.

We have recently described changes in cAMP and cGMP concentrations throughout the first cell cycle for BALB/c 3T3 cells that were arrested in the G_0 state by serum deprivation and then induced to grow by addition of fresh medium containing 20% serum (10). Fig. 1 shows the results of a similar experiment from 25 to 40 hr after medium change as the cells synchronously enter a second round of cell division. For this cell cycle analysis, serum synchronized BALB/c 3T3 cells were used, since they retain a higher degree of synchrony, even in a second round of the cycle, than any other fibroblast line. A transient 10-fold increase in cGMP was observed between 30.5 and 32 hr after medium change with a concomitant decrease in cAMP, shortly after the time of mitosis and cell division at the end of the first cycle. While cGMP did not show any other large variations, cAMP exhibited rhythmic changes during the cell cycle with low levels occurring during mitosis (26–29 hr) (21) and high levels during

TABLE 3. *Cyclic nucleotide concentrations upon addition of depleted nutrients*

Nutrient	pmol/10^6 cells		Labeled nuclei (%)
	cGMP	cAMP	
− Leucine	1.5	2.8	13
+ Leucine	1.1	2.2	30
− Methionine	1.0	2.9	4
+ Methionine	1.6	3.1	21
− Phosphate	0.5	3.5	0.3
+ Phosphate	3.9	2.0	80
− Histidine − glutamine	0.3	4.0	3
+ Histidine + glutamine	5.0	2.6	86

Swiss 3T3-4A cells were grown as described in *Methods*. After 3 days in the deficient medium [final cell densities were 1.8, 1.7, 1.8, and 1.0 × 10^6 cells per dish for cultures starved for phosphate, leucine, methionine, and both histidine and glutamine, respectively], the regular concentrations of either phosphate, leucine, methionine, or both histidine and glutamine were added back to half the cultures starved for that particular ingredient. After 20 min, cultures were isolated for cyclic nucleotide determinations from both the original starved cultures (−) and those cultures reinforced for 20 min with the added nutrients (+). Parallel cultures were also labeled with [^{3}H]thymidine from 8 until 32 hr after addition for radioautography (*Methods*).

early G_1 (about hr 30), late G_1 (32.5–34.5 hr), and S-phases (36–40 hr). The short, transient decrease in cAMP around hr 35 just before DNA synthesis was repeatedly observed. Addition of fresh medium and 20% serum to either asynchronously growing 3T3-4A or SV3T3 cultures induced no significant increase in intracellular cGMP concentrations, small reductions in cAMP (38% and 22%, respectively) within 20 min, and small increases in the overall rate of DNA synthesis (33% and 15%, respectively) (Table 4).

DISCUSSION

When nontransformed fibroblasts in tissue culture cease growing due to lack of growth substances in serum (3), the entire cell population becomes arrested at a specific point (G_0) in the cell cycle (1, 5, 15). Artificial deprivation of different low-molecular-weight nutrients in the medium, however, can arrest cells predominantly in G_0 or randomly around the entire cell cycle (13–15), although the concentrations to which the nutrients have to be lowered are probably outside the physiological range encountered in the animal (22). Our experiments then show that the early, transient changes observed in cyclic nucleotide concentrations upon growth activation of the resting cultures by restoration of the deficient nutrient are correlated with only those cell populations originally arrested at an early point in the G_0 phase. In contrast, the randomly arrested cells exhibit no significant change in cAMP or cGMP after reactivation. As the changes in cyclic nucleotides and in particular cGMP are transient phenomena which occur in less than 10% of the normal cell cycle, then only a small fraction of the cells stopped at random in the cell cycle could produce the early changes in intracellular cyclic nucleotide concentrations upon reinitiation of growth. This fraction is presumably too small to influence the overall changes. In addition, the higher cGMP levels in these randomly arrested leucine- or

TABLE 4. *Changes in cyclic nucleotide concentrations after addition of extra serum to growing cultures*

Cell type	Extra serum	cGMP (pmol/10^6 cells)	cAMP (pmol/10^6 cells)	DNA synthesis (cpm/1000 cells)	Labeled nuclei (%)
3T3-4A	−	0.80	3.7	600	64
3T3-4A	+	0.73	2.3	800	85
SV3T3	−	1.09	1.75	820	87
SV3T3	+	1.15	1.36	940	96

Swiss 3T3-4A and SV3T3 cells were grown in medium containing 10% serum for 2 days (6.2 × 10^5 cells per dish) and 4 days (8.0 × 10^6 cells per dish), respectively. Medium was removed from half the cultures and replaced by fresh medium containing 20% serum. The pmoles of cyclic nucleotides per 10^6 cells were measured 20 min after the additions for cultures grown in the old (−) and new (+) medium. Parallel cultures were also radioactively labeled from 18 to 22 hr or from 0 to 24 hr after the time of medium change for determination of DNA synthesis and labeled nuclei, respectively. DNA synthesis is recorded in cpm of [^{3}H]thymidine incorporated into DNA in 4 hr per 1000 cells or as the percentage of cells with radioactively labeled nuclei.

methionine-blocked cultures (as compared to the synchronously G_0-blocked cultures) may obscure a small increase. The DNA content per cell also indicated that the "randomly arrested 3T3 cells (by leucine and methionine deprivation) did not exactly reflect the distribution in randomly growing cells (Table 1). Similar results for intracellular cyclic nucleotide changes were observed upon reinitiation of the growth of SV3T3 cells originally arrested in a random manner by leucine deprivation (23). No conditions have yet been found to arrest these transformed cells in a reversible G_0 state. Amino-acid starvation seems to lead to cell death or a random cessation of growth (18).

The early transient changes in intracellular cAMP and cGMP concentrations were observed not only when fibroblasts in the G_0 phase of the cell cycle were induced to grow, but also when growing cells passed through the G_1 phase in a second round of cell division, while in addition cAMP concentrations exhibited rhythmic changes through the cell cycle. The time between the increase in cGMP and the maximal rate of DNA synthesis after serum addition to quiescent cultures was about 20–24 hr (10), while for a second round of cell division it was only 6–8 hr (Fig. 1), the length of the G_1 phase of the 3T3 cell cycle (24). Significantly, cGMP concentrations did not decrease to the original value of resting cultures but were approximately twice as high for about 10 hr or 2 hr, while either resting or growing cells progressed through G_0 (10) or G_1, respectively.

Recently it was found that intracellular cGMP concentrations showed a fast, transient increase after growth initiation by phytohemagglutinin in human lymphocytes (25) and by fibroblast growth factor from bovine pituitary glands in mouse fibroblasts (26). Significantly different from growth induction by serum, these highly purified growth factors induced little or no alterations in the cAMP concentration. Also, the cGMP increases in fibroblasts growing in medium containing serum were only seen in an early region of the G_1 phase of the cell cycle. Thus, if the fibroblast growth factor or a similar molecule is responsible for the fibroblast growth-

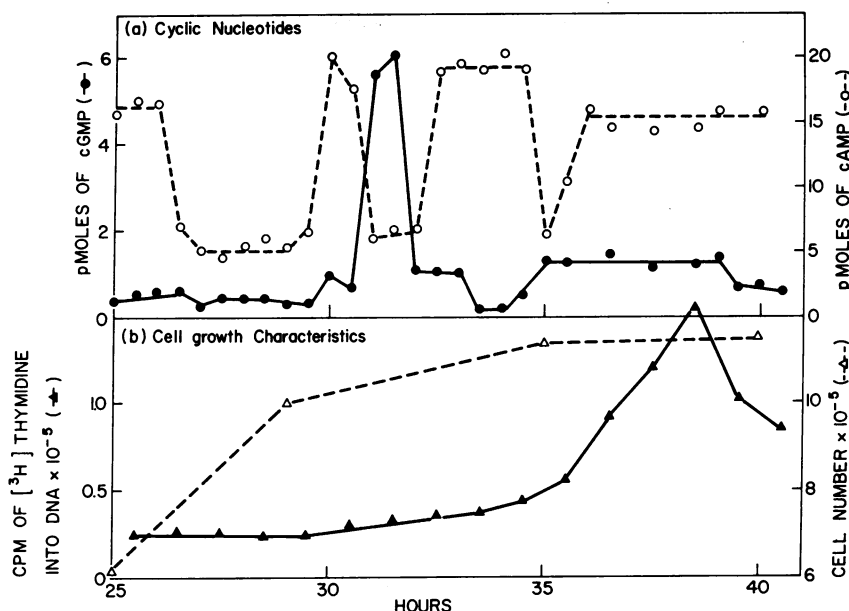

FIG. 1. Changes in cyclic nucleotide concentrations during the cell cycle. Intracellular concentrations of cAMP (O- - -O) and cGMP (●——●) (a) were measured at different times after 20% serum and fresh medium addition to resting cultures of BALB/c 3T3 cells; (b) growth of the cultures was followed by increases in cell number (Δ- - -Δ) and DNA synthesis (▲——▲). Cell cultures were grown in 3% serum and DEM and were used 4 days after termination of DNA synthesis (final cell density: 6×10^5 cells per culture; less than 0.5% of the cell nuclei became radioactively labeled with [^{3}H]thymidine during a 24-hr period immediately prior to use). Medium was removed and replaced by fresh DEM containing 20% calf serum and five petri dish cultures were isolated for cyclic nucleotide determinations at half-hour intervals, starting 25 hr after medium change. The amounts of cyclic nucleotides are expressed in pmol/mg of protein. Parallel cultures were labeled with [^{3}H]thymidine (Table 2) for 1-hr intervals and the cpm of radioactivity incorporated into DNA per culture per hr were recorded (▲——▲). During the initial round of DNA synthesis, 89% of the cell nuclei became labeled with [^{3}H]thymidine (labeling time 8–24 hr), while in the second round 65% of the cell nuclei were radioactively labeled from 32 until 45 hr and the cell population nearly doubled again to reach 2.1×10^6 cells measured at 51 hr.

initiating properties of serum, then the stimuli to initiate a new round of cell growth can only be delivered during a fraction of the total cell cycle, presumably in the early G_0 or G_1 phase. Thus, addition of serum to growing cultures of 3T3 cells failed to increase the average intracellular cGMP concentrations (Table 4). Small but significant reductions in intracellular cAMP concentrations were, however, observed and thus the capability to reduce cAMP concentrations in the cell is not so restricted during the cell cycle. Addition of extra serum to randomly growing 3T3 cultures also increases the average rate of DNA synthesis and the fraction of radioactively labeled cell nuclei in a period of 24 hr (Table 4), which is consistent with a reduction in the length of the cell cycle (probably the G_1 phase) relative to the DNA synthetic period (S phase). These results may be due to the insulin-like rather than the growth-initiating activities in serum (27), since insulin acts at physiological concentrations to reduce cAMP levels in mouse fibroblasts (26) and to shorten the length of the cell cycle in mouse mammary cells in organ cultures (28) and in some mouse fibroblastic lines (29).

Although the purified growth hormone fibroblast growth factor probably increases intracellular cGMP concentrations by stimulation of the membrane-bound guanylate cyclase in mouse fibroblasts (26), the mechanism by which some small molecular weight nutrients increase intracellular cGMP upon re-addition to starved cultures is uncertain. Perhaps starvation for certain nutrients abolishes the capability of growth factors in serum (identical or similar to fibroblast growth factor) to increase intracellular cGMP concentrations by interference with the membrane-bound guanylate cyclase or by enhanced destruction of cGMP once produced. Three possibilities could by envisaged upon starvation for the nutrient: (*1*) the generation of a negative pleiotypic program similar to the stringent response imposed on bacteria by starvation for certain amino acids (16, 17); (*2*) a failure to synthesize a critical protein in the membrane-bound guanylate cyclase system, similar to the permissive role envisaged for hydrocortisone in its potentiation of the action of the polypeptide hormone, fibroblast growth factor (29); or (*3*) direct action of the nutrient on the net production of cGMP, such as the requirement of a divalent metal ion for full activity of guanylate cyclase (P. S. Rudland, M. Hamilton, R. Hamilton, D. Gospodarowicz, and W. Seifert, submitted for publication).

In conclusion, transient cGMP increases are only associated with progression of cells through an early region of the G_0 or G_1 phase of the cell cycle, while in addition to its concomitant decrease, cAMP exhibits rhythmic variations during the cell cycle. These findings demonstrate that independent changes occur in the two intracellular cyclic nucleotide concentrations and pose the possibility that the early transient increase in cGMP could act as a specific signal for movement of cells out of the G_0 or G_1 phase of the cell cycle.

This, together with evidence reported elsewhere (10, 11, 25, 26, 29) suggests that cGMP is the intracellular activator of the positive pleiotypic response and mitogenic program of the cell, while cAMP seems to mediate the negative pleiotypic response (30). It is possible that the cell is able to read the ratio of the cyclic nucleotides, since cAMP and cGMP may interfere with each other's pathways and receptor proteins (phosphodiesterases, protein kinases, binding proteins) in an

allosteric or competitive way. We have shown in a variety of fibroblast cells (23) that the quiescent G_0 state is characterized by a very high cAMP/cGMP ratio (between 10 and 30). This ratio drops to below 1.0 in a 10 min or 20 min activated cellular state whenever cells are stimulated by serum, growth factors, or nutrients to enter the cell cycle again and to proliferate.

Transformed or malignant cells have lost the ability to rest in a G_0 state of quiescence and consequently show low cAMP/cGMP ratios under all growth conditions (23). Imbalanced cAMP/cGMP ratios were found earlier in the rapidly dividing epidermis of psoriasis (31). The increased cGMP levels of transformed cells (23) may be the consequence of an activated or relaxed guanylate cyclase system in their plasmamembranes.

The fast transient cGMP increase described here for the early G_1 or G_0 phase with its sensitivity to environmental growth stimuli may constitute the underlying molecular mechanism at the restriction point of the cell cycle (32) where the decision is made for another round of the cell cycle (delivery of the cGMP signal) or for the transition into a quiescent G_0 state characterized by high cAMP and low cGMP levels.

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