The Role of Adenosine 3':5'-Cyclic Monophosphate in the Division of WI 38 Cells

THE CELLULAR RESPONSE TO PROSTAGLANDIN E₁ AND THE EFFECTS OF AN CYCLIC ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE ANALOGUE AND PROSTAGLANDIN E₁ ON CELL DIVISION

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Inhibition of growth and DNA synthesis was observed in WI 38 cells incubated with 8-methylthioadenosine 3':5'-cyclic monophosphate or prostaglandin E₁. The effect of both compounds on cell growth was reversible. On removal of these compounds from culture media the cells initiated DNA synthesis and divided. In addition, prostaglandin E₁ stimulated cyclic AMP formation in these cells to over 40 times the normal basal value. The increase in cyclic AMP concentration in WI 38 cells after addition of prostaglandin E₁ showed a marked variation. Cells that had recently been treated with trypsin and plated at a lower cell density exhibited a smaller response to addition of prostaglandin E₁ than cells that had divided and reached confluence.

Evidence has accumulated suggesting that cyclic AMP (adenosine 3':5'-cyclic monophosphate) can influence cell division in a number of cell types in culture (Froehlich & Rachmeler, 1972; Otten *et al.*, 1971; Rozengurt & Pardee, 1972). This evidence suggests that increasing the extracellular or intracellular cyclic AMP concentration can result in a decreased growth rate. For example, the addition of dibutyryl cyclic AMP (6-*N*,2'-*O*-dibutyryl-adenosine 3':5'-cyclic monophosphate) to 3T3 mouse fibroblasts in culture arrests the division of these cells in the G₁ and G₂ phases of the cell cycle (Willingham, *et al.*, 1972). Increasing the cellular cyclic AMP concentration in the dividing neuroblastoma cells in culture inhibits their rate of division (Gilman & Nirenberg, 1971). On the other hand, a decrease in cellular cyclic AMP concentration may be associated with stimulation of cell division. Addition of serum, which is followed by increased DNA synthesis and mitotic activity, is accompanied by a decrease in cellular cyclic AMP (Otten *et al.*, 1972). Burger *et al.* (1972) reported a temporary fall in the cellular cyclic AMP concentration during the onset of mitosis. Certain agents which are known to act on the cell surface, such as Pronase, can initiate cell division in fibroblast cultures. Bombik & Burger (1973) have shown that the action of such agents can be blocked by the addition of dibutyryl cyclic AMP. These observations suggest that alterations in cyclic AMP concentrations can affect cell division and that certain

extracellular conditions may control cell division by affecting the membranal components of the cyclic AMP system, i.e. the adenylate cyclase. It may be that only temporary changes in cyclic AMP concentrations of cells are necessary in controlling cell growth.

The present report deals with the role of cyclic AMP in cell division in a diploid human embryonic cell line, WI 38, in culture. An attempt was made to inhibit cell division by addition of an analogue of cyclic AMP, 8-methylthio cyclic AMP (8-methylthioadenosine 3':5'-cyclic monophosphate) to the cultures. We also attempted to inhibit cell division by raising the cellular cyclic AMP concentration with prostaglandin E₁ in the presence of a phosphodiesterase inhibitor, aminophylline. Cyclic AMP concentrations were determined in cells that were growing exponentially as well as in cells that had divided and reached confluency. The ability of prostaglandin E₁ to elevate the cyclic AMP concentration in both growing and confluent cultures was also examined.

Materials and Methods

Materials

Cyclic AMP and aminophylline were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. 8-Methylthio cyclic AMP was provided through the generosity of S. Hess, Squibb Institute for Medical Research, Princeton, N.J., U.S.A. Prostaglandins

E₁, E₂ and F_{2a} were a gift from J. Pike, Upjohn Co., Kalamazoo, Mich., U.S.A. The reagents for the radioimmunoassay were made available from Collaborative Research Corp., Waltham, Mass., U.S.A. [³H]Thymidine (specific radioactivity 20 Ci/mmol) and cyclic [³H]AMP (specific radioactivity 20–30 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Cell culture

WI 38 human diploid fibroblasts were obtained at passage 15 from Dr. L. Hayflick (Hayflick & Moorhead, 1961). The cells were serially grown in 80 cm² Falconware (Fisher Scientific Co., Medford, Mass., U.S.A.). The cultures were maintained at 37°C in a humidified incubator gassed with CO₂+air (5:95). The growth medium was composed of Ham's Formula F-10 (Ham, 1963) (Grand Island Biological Co., Grand Island, N. Y., U.S.A.), supplemented with 10% foetal calf serum (Grand Island Biological Co.; Virus Screened), streptomycin (50 units/ml), penicillin (50 units/ml) and amphotericin B (2.5 µg/ml). All experiments were carried out on cells before passage 30 (Hayflick & Moorhead, 1961). Feeding was performed as a routine every 3–4 days unless otherwise stated.

Cell incubation and assay for cyclic AMP content

The removal of media from the cultures followed by addition of fresh media can cause changes in cellular functions (Todaro *et al.*, 1965). Therefore changes of media for feeding of cultures were made at least 24 h before the beginning of the experiment. After the addition of the hormone to the cell media, the cell cultures were incubated as a routine for 15 min at 37°C in CO₂+air (5:95) in a humidified incubator. Control cultures received an equivalent volume of media without hormone present. The incubation was stopped by the removal of the media from the cells by suction, followed by the addition of 1 ml of 7% (w/v) HClO₄ to the cell monolayer, and the cells were collected by scraping with a rubber 'policeman'. The HClO₄ solution contained 10000 d.p.m. of cyclic [³H]AMP (0.1 pmol) as a tracer to enable a determination to be made of the recovery of cyclic AMP from the sample. Cells that were kept at 0–4°C were then centrifuged at 1000g for 30 min. The supernatant was neutralized with 3 M-KOH and the resulting insoluble salt was removed by centrifugation. The supernatant was then applied to a Dowex 50 column (6 mm × 35 mm), eluted with water and the cyclic AMP fraction collected and freeze-dried (Polgar *et al.*, 1973). The product was resuspended in an appropriate volume of water. Samples were taken for estimating recovery and for assay by the radioimmunoassay (Steiner *et al.*, 1969). Since the cells were not washed

before the addition of the HClO₄, 7% (w/v), the residual medium might have affected the accurate measurement of cyclic AMP. This possibility was excluded by assaying the medium in contact with cells. The results indicated that the medium did not interfere in the radioimmunoassay. Exogenous cyclic AMP added to the medium alone and to the samples was recovered. A standard curve was performed with each assay. The most linear portion of the curve was found in the range of 1–50 pmol of cyclic AMP. Samples were appropriately diluted to be within this range.

DNA determination

The DNA determinations were carried out, after the cells had been collected by centrifugation at 1000g for 30 min, by digesting the cell pellets in 0.5 M-HClO₄ and assaying the resulting supernatant by the diphenylamine method (Burton, 1956).

DNA synthesis

Cells were incubated for 1 h with 5 µCi of [³H]-thymidine. The medium was aspirated, cells were washed with 0.9% NaCl and 5% (w/v) trichloroacetic acid was added. The precipitable cell material was collected by scraping with a rubber policeman and filtered through a Millipore filter (Millipore Corp., Bedford, Mass., U.S.A.; pore size 0.45 µm). The filter containing the precipitate was then placed in a scintillation vial and treated with 1 ml of methoxyethanol and 4 ml of scintillation fluid [0.3 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 5 g of 2,5-diphenyloxazole and 25% (w/v) methoxyethanol in 1 litre of toluene]. Radioactivity was determined in a Packard Tri-Carb liquid-scintillation counter. The quenching in various fractions showed negligible variation, obviating the need for correction.

Results

Confluent cultures of density-inhibited WI 38 cells can be stimulated to divide when the nutritional conditions are changed (Rovera & Baserga, 1971). Fig. 1 shows the effect of fresh media containing foetal calf serum on the proliferative activity of the WI 38 cells. At the time that the fresh medium was added, the cells exhibited low DNA synthesis. DNA synthesis increased and reached a peak at 16–20 h after the addition of fresh serum. An increase in cell number occurred 25 h later.

The addition of a cyclic AMP derivative, dibutyryl cyclic AMP, to the culture media has been reported to induce a decrease in the final saturation density of the cell culture (Johnson *et al.*, 1971). However, the nature of this action has been controversial because of a possible parallel effect of the butyryl side-chain portion of the molecule (Wright, 1973). To exclude the possible effects of the butyryl component of

Fig. 1. DNA synthesis and cell division in confluent monolayers of WI 38 cells after change of medium

Cells were grown for 6 days with the medium changed 48 h after plating. After 6 days the medium was changed, fresh medium added and cells were incubated at the times specified for 1 h with 5 μ Ci of [³H]thymidine. Each point represents an average of duplicate cultures. ●, [³H]-Thymidine (c.p.m./10⁶ cells); ○, 10⁻⁶ × no. of cells/plate.

Table 1. Effect of 8-methylthio cyclic AMP on the initiation of DNA synthesis in confluent cultures

Cells were plated at 2 × 10⁵/20 cm² plate and grown until confluent (2 × 10⁶/20 cm² plate). Initial [³H]thymidine incorporation was 150 c.p.m./2 × 10⁶ cells before addition of compounds plus fresh medium. DNA synthesis was followed as a function of time after the removal of medium and the addition of fresh medium plus serum with or without the analogue, followed by incubation of cells for 1 h as described in the Materials and Methods section.

8-Methylthio cyclic AMP added (mM)	Time ...	Incorporation (c.p.m./2 × 10 ⁶ cells)	
		21 h	41 h
0.00		2187, 1312	202, 272
1.00		153, 147	129, 115
0.01		1129, 1172	250, 491

dibutyryl cyclic AMP, we studied the effect of a cyclic AMP analogue, 8-methylthio cyclic AMP. This compound inhibited DNA synthesis effectively in the WI 38 cell line. Table 1 shows that the addition of 8-methylthio cyclic AMP to the cultures after serum stimulation caused a marked inhibition of DNA synthesis 21 h after addition of both analogue and serum. After 41 h [³H]thymidine incorporation

in both control and treated cultures declined, reflecting an expected cessation of DNA synthesis. Lower concentrations (0.01 mM) of the analogue were not as effective in inhibiting serum-stimulated DNA synthesis as was the higher concentration (1.00 mM).

Table 2 demonstrates that the effect of 8-methylthio cyclic AMP on DNA synthesis is reversible. Expt. B shows that the addition of 1.00 mM-8-methylthio cyclic AMP to confluent cultures at the time of stimulation with fresh serum resulted in inhibition of DNA synthesis and cell division. The continued presence of 8-methylthio cyclic AMP in the medium beginning 72 h before stimulation with fresh serum resulted in a measurable decrease in DNA synthesis as well as a retarded increase in total cell number (Expt. C). Removal of 8-methylthio cyclic AMP, which was present in the culture for 72 h, followed by immediate stimulation with fresh serum, resulted in increased cellular DNA synthetic capacity and massive cell division (Expt. D).

It has been proposed that the addition of 8-methylthio cyclic AMP to the cultures resulted in its entry into the cell, which in effect leads to increased cellular cyclic AMP concentration (Muneyama *et al.*, 1971). Increasing intracellular cyclic AMP by means of hormonal stimulation of adenylate cyclase should prove to be equally effective in inhibition of DNA synthesis. To investigate this possibility, the effect of several hormones on cyclic AMP formation in these cells was determined and correlated with DNA synthesis. The effect on cyclic AMP concentrations in cells when different concentrations of prostaglandins E₁, E₂ and F_{2 α} , noradrenaline, glucagon and isoproterenol were added to cultures is shown in Table 3. Of the agents tested, prostaglandin E₁ proved to be the most potent cyclic AMP stimulator. Maximal stimulation with prostaglandin E₁ was observed at a concentration of 50 μ M, with a gradually decreasing response to near-control concentrations at 5 μ M. The prostaglandin E₁-stimulated increase in cyclic AMP concentration in the presence of aminophylline was about twice that with prostaglandin E₁ alone. Prostaglandin E₂ was not as potent a stimulator at 0.1 mM and increased the cyclic AMP concentration only threefold over control values. Prostaglandin F_{2 α} was inactive, whereas glucagon, noradrenaline and isoproterenol produced relatively small responses.

The time-course of stimulation with prostaglandin E₁ (Fig. 2) showed a maximal stimulation after 5 min. The elevated cyclic AMP concentrations remained essentially unchanged for 30 min after stimulation. By 16 h, however, the high intracellular cyclic AMP concentrations were appreciably decreased. The presence of aminophylline in the culture did not alter the decline of cyclic AMP concentration.

Prostaglandin E₁ stimulation of cyclic AMP production in these cells appeared to be related to cell density. Fig. 3 shows the cellular response to

Table 2. *Effect of the cyclic AMP analogue 8-methylthio cyclic AMP on DNA synthesis and cell division*

At 24 h after plating, cells were incubated for 72 h with (+) or without (–) 8-methylthio cyclic AMP (1.00 mM). At zero time, medium was removed from all plates and cells were restimulated by the addition of fresh medium with (+) or without (–) 8-methylthio cyclic AMP. The radioactivity represents the [³H]thymidine incorporation into the acid-insoluble cell fraction per plate. Each value is the average of duplicate determinations. Expt. A, no analogue added; Expt. B, analogue added at zero time along with fresh medium; Expt. C, analogue added 72 h before stimulation and at zero time; Expt. D, analogue added 72 h before restimulation and removed at zero time.

Expt.	72h before restimulation		Time after restimulation ... 0		24h	48h		51h
	8-Methylthio cyclic AMP	No. of cells/plate	8-Methylthio cyclic AMP	No. of cells/plate	Radioactivity (c.p.m./plate)	No. of cells/plate	Radioactivity (c.p.m./plate)	Radioactivity (c.p.m./plate)
A	–	2.4×10^5	–	6.6×10^5	1313 ± 262	13.4×10^5	308 ± 51	192 ± 21
B	–	2.4×10^5	+	6.6×10^5	138 ± 22	5.1×10^5	135 ± 15	39 ± 8
C	+	2.4×10^5	+	3.6×10^5	242 ± 24	6.6×10^5	93 ± 11	157 ± 18
D	+	2.4×10^5	–	3.6×10^5	514 ± 48	9.6×10^5	659 ± 101	1170 ± 117

Table 3. *Stimulation of cellular cyclic AMP production by various compounds*

The cultures were plated 6 days before the start of the experiment. Incubations were carried out for 15 min in the presence of prostaglandin E₁, E₂ or F_{2α}, noradrenaline, isoproterenol, glucagon, or medium alone (control). Duplicate values for incubations are shown.

Additions	Cyclic AMP (pmol/μg of DNA)
None (control)	2.7
	1.8
Prostaglandin E ₁ (0.1 mM)	22.0
	28.0
(50 μM)	35.7
	40.8
(10 μM)	25.2
	23.0
(5 μM)	6.4
	5.7
Prostaglandin E ₂ (0.1 mM)	7.1
	6.9
(10 μM)	2.6
	2.6
(1 μM)	4.0
	2.2
Noradrenaline (50 μM)	4.2
	5.2
Isoproterenol (50 μM)	8.7
	6.1
Prostaglandin F _{2α} (1 μM)	2.3
	1.1
Glucagon (5 μM)	2.5
	4.4

addition of prostaglandin E₁ at 1–7 days after plating. The cyclic AMP response to prostaglandin E₁ (50 μM) increased with increasing cell density to a maximum at 4 days. Cyclic AMP concentrations in

Fig. 2. *Production of cyclic AMP with time after stimulation by prostaglandin E₁*

Cells grown to 2×10^6 cells/plate were stimulated with 50 μM-prostaglandin E₁ and samples were collected at the times indicated. Values indicate the averages of duplicate determinations; the variation between duplicates was never greater than 10%. Cultures receiving an equal amount of fresh medium alone served as control. ●, Control cultures; ○, prostaglandin E₁-stimulated cultures.

unstimulated cultures remained virtually unchanged throughout the 7 days.

Because of its effectiveness in stimulating cyclic AMP formation prostaglandin E₁ was used to determine whether a prostaglandin E₁-induced increase in cellular cyclic AMP concentrations would also inhibit DNA synthesis. To obtain a maximal increase in cyclic AMP concentration, cultures were used as they approached confluence. Effective inhibition of DNA synthesis by prostaglandin E₁ in the presence of aminophylline is illustrated in Fig. 4. An increase in DNA synthesis in the control cultures occurred 12 h after serum stimulation. This increase was appreciably less in the cells exposed to prostaglandin E₁ (50 μM)

Fig. 3. Prostaglandin E_1 stimulation in cultures at various times after plating

Cells were plated at a density of 10^4 cells/cm². At indicated times after plating, samples were incubated for 15 min with prostaglandin E_1 (final concn. $50\mu\text{M}$). Control cultures were treated similarly but only medium was added. Cyclic AMP concentrations are reported per μg of DNA. Cultures were pooled where necessary. All experiments were done in duplicate. A change of medium, with fresh medium added to cultures, was carried out so that cells were fed at least 48 h before incubation. ●, Control cultures; ○, prostaglandin E_1 -stimulated cultures.

Fig. 4. DNA synthesis in the WI 38 cultures in presence of prostaglandin E_1 and aminophylline

Cells were plated at 2×10^4 cells/cm², fresh medium was added 24h later and then cells were incubated for 3 days. The experiment was initiated when the culture medium was replaced with fresh serum-containing medium. Prostaglandin E_1 ($50\mu\text{M}$) and aminophylline (0.5mM) were included in the fresh medium as indicated. At specified times cultures were incubated with $5\mu\text{Ci}$ of [^3H]thymidine for 1 h and the ^3H incorporation into DNA was determined. Cultures receiving fresh serum-containing medium alone served as controls. ●, Control cultures; ○, cultures receiving 0.5mM -aminophylline; ▲, cultures receiving 0.5mM -aminophylline plus $50\mu\text{M}$ -prostaglandin E_1 . Values shown represent c.p.m. of [^3H]thymidine/plate.

and aminophylline (0.5mM). After 24h the difference in DNA synthesis between prostaglandin E_1 -treated and untreated cultures was even more marked. Aminophylline (0.5mM) alone was not effective in decreasing DNA synthesis in these cells.

Discussion

The WI 38 cell line was established from embryonic human lung tissue and has a finite life span *in vitro* which has been related to the number of mitotic divisions (Hayflick & Moorhead, 1961). The cells maintain a normal karyotype and growth pattern until the onset of their degenerative period, at which point an increasing proportion of cells fall out of the division cycle (Macieira-Coelho *et al.*, 1966). The reversibility of the effect of 8-methylthio cyclic AMP on DNA synthesis and cell growth and the lack of cytotoxicity observed with this cyclic AMP analogue suggest that it is capable of modulating cell growth and division in much the same way as intracellular cyclic AMP. The observation that these cells contain an active cyclic AMP system, highly responsive to prostaglandin E_1 stimulation, was further evidence of this possibility. In fact, the addition of prostaglandin E_1 and aminophylline to the cultures at a prostaglandin E_1 concentration known to elevate cyclic AMP concentrations in these cells resulted in a pattern of growth inhibition and cessation of DNA synthesis similar to that observed with 8-methylthio cyclic AMP. We thus demonstrated that both the addition of exogenous cyclic AMP and substantial increases in intracellular cyclic AMP by hormone stimulation of adenylate cyclase can affect cell division in this stable diploid line. However, the precise nature of the actual role of this system in the process of cell division still remains to be determined. One consideration in interpreting the effects of prostaglandin E_1 on cell division is the possibility that prostaglandin E_1 might inhibit cell division through some mechanism other than adenylate cyclase stimulation. In addition, our findings show that although the elevation of intracellular cyclic AMP concentrations inhibited DNA synthesis in these cells, basal cyclic AMP concentrations did not increase as cell division decreased. In fact, cellular cyclic AMP concentrations remained constant throughout cell growth and at confluence, when cell division was slowed markedly. One explanation for the lack of change in cyclic AMP concentrations may be inherent in using a non-synchronized cell culture to study a relationship which might be dependent on discrete events occurring at specific times in the cell cycle (Burger *et al.*, 1972). In the WI 38 cell line a degree of synchrony is achieved as the cultures reach a confluent monolayer (Rovera & Baserga, 1971). The arrest of phase II WI 38 cells at confluence has been reported to occur in the G_1 and G_2 phases of the cell cycle

(Macieira-Coelho *et al.*, 1966). The confluent cells should therefore exhibit an increase in cyclic AMP concentration if an increase occurred as a natural correlation to the cessation of division. A comparison of basal cyclic AMP concentration during the lag phase, the exponential phase and the final contact-inhibited state showed no substantial difference. In additional experiments (M. Kurtz, P. Polgar & A. Rutenburg, unpublished work) confluent cells were stimulated by refeeding with the fresh serum-containing medium. A temporary decrease in cyclic AMP basal concentration was observed. This observation demonstrates that transient changes in cyclic AMP concentrations do occur in WI 38 cells, suggesting that temporary changes in cyclic AMP concentrations may act as one means of controlling division in this cell line.

Although basal cyclic AMP concentrations did not reflect the state of cell division in the WI 38 cell line, the relative change in cyclic AMP concentrations consequent to stimulation of adenylate cyclase by prostaglandin E_1 did parallel mitotic activity. This was reflected by an increase in cyclic AMP concentrations with prostaglandin E_1 stimulation as cultures increased in density and DNA synthesis per cell decreased. Alteration of the cell surface can result in stimulation of cell division. This type of stimulation can be counteracted by exogenously added cyclic AMP. For example, Bombik & Burger (1973) have reported that Pronase-induced DNA synthesis can be blocked by the addition of dibutyryl cyclic AMP.

Adenylate cyclase and the receptors which control much of the activity of this enzyme system are located, at least in mammalian tissue, within the plasma membrane. The location of this enzyme system makes it well suited as a cellular control element for certain extracellular impulses. Membranal modifications, which are known to occur during the cell cycle, differentiation and transformation, may alter the specificity of the receptor and/or the activity of the adenylate cyclase, thus altering the cyclic AMP response to external stimuli. Makman (1971) has illustrated this point with particulate preparations showing increasing adenylate cyclase activity and adenylate cyclase response to hormones as cell density increased in cultures of 3T3 mouse fibroblasts. Schwartz *et al.* (1973) also showed a similar change in adenylate cyclase responsiveness with increasing cell density.

Our observation that increased time in culture and/or increased cell density is correlated with an increasingly responsive adenylate cyclase system may be related to a degree of damage done to the membrane by the trypsin treatment of the cells for subculture. Conceivably, the repair of the membrane could be correlated with increasing responsiveness to prostaglandin E_1 stimulation as density increased. This type of membrane repair has been reported for

the insulin effector system in isolated fat-cells (Kono, 1969). The restoration of the proper membrane structure could then explain the increased response to prostaglandin E_1 .

A more intriguing explanation for these results would be that at certain stages in the cell cycle, or as cell density increases, the response of adenylate cyclase to external stimuli may be so altered as to permit the cyclic AMP system to act as a controlling element in cellular division. It remains to be determined whether changes in response to prostaglandin E_1 stimulation also occur during the cell cycle.

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