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REGULATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY IN HUMAN LUNG FIBROBLASTS

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Summary

Cyclic nucleotide phosphodiesterase activity (3′,5′-cyclic-nucleotide 5′-nucleotidohydrolase, 3.1.2.17) was studied in homogenates of WI-38 human lung fibroblasts using 0.1—200 μM cyclic nucleotides. Activities were observed with low K_m for cyclic AMP (2—5 μM) and low K_m for cyclic GMP (1—2 μM) as well as with high K_m values for cyclic AMP (100—125 μM) and cyclic GMP (75—100 μM). An increased low K_m cyclic AMP phosphodiesterase activity was found upon exposure of intact fibroblasts to 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase activity in broken cell preparations, as well as to other agents which elevate cyclic AMP levels in these cells. The enhanced activity following exposure to 3-isobutyl-1-methylxanthine was selective for the low K_m cyclic AMP phosphodiesterase since there was no change in activity of low K_m cyclic GMP phosphodiesterase activity or in high K_m phosphodiesterase activity with either nucleotide as substrate. The enhanced activity due to 3-isobutyl-1-methylxanthine appeared to involve de novo synthesis of a protein with short half-life (30 min), based on experiments involving cycloheximide and actinomycin D. This activity was also enhanced with increased cell density and by decreasing serum concentration. Studies of some biochemical properties and subcellular distribution of the enzyme indicated that the induced enzyme was similar to the non-induced (basal) low K_m cyclic AMP phosphodiesterase.

Introduction

The intracellular levels of cyclic AMP are determined and may be regulated both by synthesis of this nucleotide catalyzed by adenylate cyclase and by degradation to 5′-AMP via the action of cyclic nucleotide phosphodiesterase(s).

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Various cell types contain more than one enzyme or form of enzyme(s) capable of degrading cyclic AMP and/or cyclic GMP and these activities may be distinguished on the basis of K_m for cyclic nucleotides [1], differences in specificity for cyclic AMP and cyclic GMP [2] and requirement for a Ca^{2+}-binding protein [3].

Also, exposure of 3T3 and SV-40 virus-transformed 3T3 cells [1], chick embryo fibroblasts [4], L929 cells [5] as well as rat astrocytoma cells [6,7] to cyclic AMP, its analogues or agents which elevate intracellular levels of cyclic AMP, results in increased cyclic nucleotide phosphodiesterase activity.

We report here a study of cyclic nucleotide phosphodiesterase activity in human diploid (WI-38 strain) fibroblasts and an increased low K_m cyclic AMP activity found upon exposure of intact fibroblasts to 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase activity in broken cell preparations. Evidence is presented indicating that the enhanced activity is associated with de novo synthesis of a protein with a relatively short half life and that activity may also be regulated by cell density and serum concentration. Part of this work has been presented in a preliminary communication [8]. We also present some comparative studies for SV-40 virus transformed WI-38 and C-6 astrocytoma cells.

Materials and Methods

Strain WI-38 and SV-40 virus transformed WI-38 (VA 13B 2RA) human lung fibroblast cells (obtained from Dr. L. Hayflick) were grown on 60-mm Falcon plastic tissue culture dishes using Eagle's basal medium supplemented with 10% fetal calf serum in an atmosphere of 95% air and 5% CO_2. Rat C-6 astrocytoma cells were grown in Dulbecco's medium as previously described [9]. Unless otherwise mentioned cells were grown to a density of 500—600 μg protein/60 mm dish. For each experiment the medium was removed from the culture dishes, 1 ml of complete medium added, and the cells equilibrated at 37° C for 2 h prior to addition of agent(s) as indicated. After treatment with agent(s), cells were washed 4 times with chilled phosphate buffered saline prior to removal from the plates and homogenization. Washed cells from each 60-mm plate were usually taken up in 0.5 ml 40 mM Tris · HCl buffer (pH 8.0)/10% sucrose and homogenized for 10—12 strokes in an all-glass Dounce homogenizer. In experiments with low density cells, 3—5 plates were generally pooled and taken up in 0.5 ml buffer. Experiments involving mixing of enzyme from cells exposed to 3-isobutyl-1-methylxanthine with enzyme from control cells indicated that the final diluted enzymes did not contain the inhibitor in a concentration range sufficient to inhibit directly phosphodiesterase.

For assay of cyclic nucleotide phosphodiesterase activity [10], the 250 μl reaction mixture contained 10 mM Tris · HCl buffer (pH 8.0)/3.75 mM 2-mercaptoethanol/10 mM $MgCl_2$/100 μg equivalent of cell homogenates as a source of phosphodiesterase/25 μg of *Ophiophagus hannah* venom as a source of nucleotidase and appropriate substrate concentration of ^3H-labeled cyclic nucleotides. Prior to use as substrate both [^3H]cyclic AMP and [^3H]cyclic GMP (New England Nuclear Co) were purified on BioRad AG1-X8 formate columns and the formic acid eluates lyophilized, reconstituted in water and stored at

—20°C for up to 2—3 weeks. Samples containing approx. 30 000 cpm were used were used in each incubation. Substrate concentrations were 5 μM cyclic AMP or 1 μM cyclic GMP for the low K_m phosphodiestrease assays and 100 μM cyclic AMP or cyclic GMP for the high K_m assays. The reaction mixture was incubated at 30°C for 3 min. The reaction was stopped by adding 1 ml BioRad AG1-X8 resin (1 : 3 w/v slurry in water) to remove unreacted cyclic AMP or cyclic GMP. A 200-μl aliquot of supernatant was removed for counting in a liquid scintillation spectrometer. Specific activity of phosphodiesterase was expressed as pmol of cyclic AMP or cyclic GMP degraded/min per mg protein. The venom enzyme caused essentially complete conversion of 5'-AMP or 5'-GMP to adenosine or guanosine and did not inhibit phosphodiesterase activity. Proteins were determined by the method of Lowry et al. [11]. Cyclic AMP, cyclic GMP and Snake venom were from Sigma Chemical Co., 3-isobutyl-1-methylxanthine from Aldrich and BioRad AG-X8 resin from Bio Rad Co.

Results

Multiple K_m values for phosphodiesterase in human lung fibroblasts

Cyclic nucleotide phosphodiesterase activity was studied in homogenates of WI-38 human lung fibroblasts in assays using 0.1—200 μM substrate. The low K_m for cyclic AMP was found to be between 2—5 μM, and for cyclic GMP, 1—2 μM: whereas, the high K_m enzyme for cyclic AMP was between 100—125 μM and for cyclic GMP, 75—100 μM (Fig. 1 and inset). Thus, there appears to be both low and high K_m enzymes or enzyme forms for both cyclic AMP and cyclic GMP.

Effects of various agents on phosphodiesterase activity of cultured cells

The low K_m cyclic AMP phosphodiesterase activity in the whole homogenate increased when WI-38 cells were treated with 3-isobutyl-1-methylxanthine for 24 h (Table I). This increase appeared to be selective for the low K_m enzyme for cyclic AMP with essentially no change in the high K_m enzyme activity for cyclic AMP or either low or high K_m activity for cyclic GMP phosphodiesterase (Table I). Agents which were previously shown to increase the intracellular level of cyclic AMP, for example epinephrine, prostaglandin E_1, adenosine, 2-chloroadenosine or theophylline were also tested and found to increase the low K_m cyclic AMP phosphodiesterase activity in the WI-38 cell homogenates (Table I). In additional studies (unpublished data), the selective increment of the low K_m activity was evident when cyclic AMP substrate concentrations were varied over a wide range (5—200 μM) as used in the experiments of Fig. 1.

The influence of 3-isobutyl-1-methylxanthine on phosphodiesterase activity was also examined in SV-40 transformed WI-38 fibroblasts, and in C-6 rat astrocytoma cells. Selective increases in low-K_m phosphodiesterase activity occurred in both the SV-40 transformed human fibroblasts and the rat astrocytoma cells upon exposure of the intact cells to this agent (Table I). The magnitude of the increase was similar to that obtained with the normal WI-38 cells. Following the addition of 3-isobutyl-1-methylxanthine to WI-38 cells, after a short lag phosphodiesterase activity increased rapidly, reaching maximal levels by 6 h

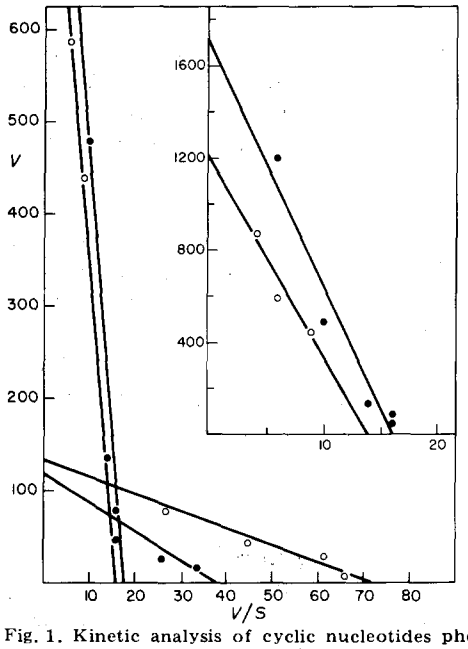

Fig. 1. Kinetic analysis of cyclic nucleotides phosphodiesterase activity of WI-38 cells: Cells were harvested at a density yielding 400—500 mg protein per 60-mm culture dish, homogenized and the whole homogenates assayed as described in the text. Initial velocity, V, is plotted versus the ratio V/S (Eadie-Hofstee Plot), where S represents either the concentrations of cyclic AMP (●———●) or cyclic GMP (○———○) present as substrate. Figure shows data for substrate concentrations in range of 0.5—50 μM cyclic AMP and 0.1—75 μM cyclic GMP, from which K_m values of 3 μM for cyclic AMP and 2 μM for cyclic GMP are derived. Insert shows data for substrate concentration of 5—200 μM cyclic AMP and 50—200 μM cyclic GMP from which K_m values of 106 μM for cyclic AMP and 86 μM for cyclic GMP are derived.

(Table II). This high level was maintained even after 24 h if 3-isobutyl-1-methylxanthine was present in the medium (Table II). Also, as little as 5 μM 3-isobutyl-1-methylxanthine brought about a significant increase in the activity of phosphodiesterase (Table II). The effect was maximal at 250 μM and this concentration was used in most of the present studies. It may be noted that 3-isobutyl-1-methylxanthine at concentrations up to 500 μM had no evident effect on cell viability, growth, or morphology.

De novo induction of phosphodiesterase

It has been reported for other cells that the increase in the phosphodiesterase activity associated with an increase in the intracellular levels of cyclic AMP can be inhibited by actinomycin D and cycloheximide [1,4,5,7]. In WI-38 cells we also find that the enhancement of phosphodiesterase activity by 3-isobutyl-1-methylxanthine is prevented by the presence of either cycloheximide or actinomycin D suggesting that the process involves or requires de novo synthesis of protein as well as RNA synthesis (Table II).

Loss of induced phosphodiesterase activity following removal of 3-isobutyl-1-methylxanthine

The stability of the increased enzyme activity has been studied in WI-38 cells

TABLE I

PHOSPHODIESTERASE ACTIVITY OF CULTURED CELLS FOLLOWING EXPOSURE TO VARIOUS AGENTS

Phosphodiesterase activity of homogenates was measured using 5 μM and 100 μM cyclic AMP respectively as substrate. Values represent means ± standard deviations of three experiments. In each experiment, 3 plates were used and homogenized separately for each condition studied with duplicate determinations carried out for each. Cells were grown to a density at which the average protein concentration was 450–500 μg for WI-38 cells and 600–800 μg for the other two cell types per 60-mm Falcon dish. For each experiment 2 h prior to the addition of any agent, the medium was removed from the plates, fresh medium with 10% fetal calf serum was added. Incubations with the agents were for 24 h.

Cells	Treatment (μM)	Phosphodiesterase activity (pmol cyclic nucleotide breakdown/min per mg protein) with suhbstrate:	
		5 μM cyclic AMP	100 μM cyclic AMP
WI-38	Control	100 ± 10	1680 ± 450
	3-isobutyl-1-methylxanthine (250)	220 ± 13	1695 ± 40
	Theophylline (250)	160 ± 15	1672 ± 300
	Adenosine (70)	160 ± 10	1660 ± 330
	2-chloroadenosine (125)	180 ± 21	1690 ± 200
	Epinephrine (1)	138 ± 9	1645 ± 85
	Prostaglandin (7)	170 ± 20	1682 ± 310
C6-rat astrocytoma	Control	300 ± 20	1367 ± 150
	3-isobutyl-1-methylxanthine (250)	470 ± 40	1400 ± 45
SV-40 transformed WI-38	Control	149 ± 17	1250 ± 300
	3-isobutyl-1-methylxanthine (250)	242 ± 43	1270 ± 300
		1 μM cyclic GMP	100 μM cyclic GMP
WI-38	Control	60 ± 10	1000 ± 240
	3-isobutyl-1-methylxanthine (250)	60 ± 9	950 ± 100

which were first treated with 3-isobutyl-1-methylxanthine for 6 h. The inhibitor was then removed by washing cells with medium 4 times. Finally 1 ml of fresh medium was added back to the cells with or without cycloheximide also present. Cells were harvested at various times and assayed for the induced enzyme (enhanced enzyme) activity (Fig. 2). A small (15–30 min) lag occurred before loss of activity was evident when the medium did not contain cycloheximide. This may indicate that immediate reversal of the effect of the inducer on cyclic AMP levels did not occur with the washing procedure. The half-life of the induced enzyme was found to be around 65–75 min in absence of cycloheximide, whereas in presence of the later agent the half-life was only 30 min. It is apparent from Fig. 2 that the induced enzyme is unstable in nature. After about 6 h the activity was not significantly elevated over the basal level (unpublished data).

Effect of cell density on induction of cyclic AMP phosphodiesterase

The activity of the low-K_m cyclic AMP phosphodiesterase first increased as WI-38 cells grew from sparse to a moderate cell density and then decreased with further increase in cell density (Table III). Similar changes with cell

TABLE II

INFLUENCE OF TIME OF EXPOSURE AND CONCENTRATION OF 3-ISOBUTYL-1-METHYLXANTHINE AND EFFECT OF CYCLOHEXIMIDE AND ACTINOMYCIN D ON INDUCTION OF PHOSPHODIESTERASE ACTIVITY IN WI-38 CELLS

Control (basal) phosphodiesterase activity was 160 ± 25 pmol cyclic nucleotide breakdown/min per mg protein. Values represent mean percent increase above basal activity ± standard deviations of three experiments assayed as explained in Table I with 5 μM cyclic AMP as substrate. Cells were treated with 3-isobutyl-1-methylxanthine at the concentrations and for the time periods indicated, either without or with actinomycin D (2 μg/ml) or cycloheximide (10 μg/ml) added 30 min prior to the addition of 3-isobutyl-1-methylxanthine.

Cells treated with	Concentration (μM)	Time (h)	Percent increase above control phosphodiesterase activity
3-isobutyl-1-methylxanthine	250	0	0 ± 14
	250	0.25	0 ± 5
	250	0.50	1 ± 3
	250	1	9 ± 5
	250	2	29 ± 7
	250	3	82 ± 4
	250	6	107 ± 8
	250	24	108 ± 9
	5	6	50 ± 8
	50	6	70 ± 5
	100	6	72 ± 3
	250	6	107 ± 8
	500	6	113 ± 9
Actinomycin D		7	−2 ± 10
Cycloheximide		7	−7 ± 16
3-isobutyl-1-methylxanthine	150	6.5	106 ± 16
3-isobutyl-1-methylxanthine plus actinomycin D	250	6.5 7	+1 ± 12
3-isobutyl-1-methylxanthine plus cycloheximide	250	6.5 7	0 ± 16

density occurred for high-K_m cyclic AMP phosphodiesterase and for cyclic GMP phosphodiesterases measured at both low and high substrate concentration (unpublished data). While these changes with cell density were not large in magnitude, they were consistently obtained in a large number of experiments. Induction of phosphodiesterase by 3-isobutyl-1-methylxanthine is partly influenced by the cell density. The inducing ability is found to be maximum at a very low cell density (sparse) whereas the lowest level of induction was obtained at a medium high density (prior to confluency) (Table III). Since at medium high density these cells have a relatively high level of endogenous cyclic AMP [12] as well as phosphodiesterase, the extent of induction of phosphodiesterase at that time may in fact be limited by the basal level of cyclic AMP already present in the cells.

Effect of low serum on induction of cyclic AMP phosphodiesterase

When low density WI-38 cells were grown in absence of serum or with serum concentrations below that needed for cell proliferation, the levels of cyclic GMP could be maintained at a relatively high level. Under this condition cyclic AMP levels are also increased [13]. The basal low K_m cyclic AMP phospho-

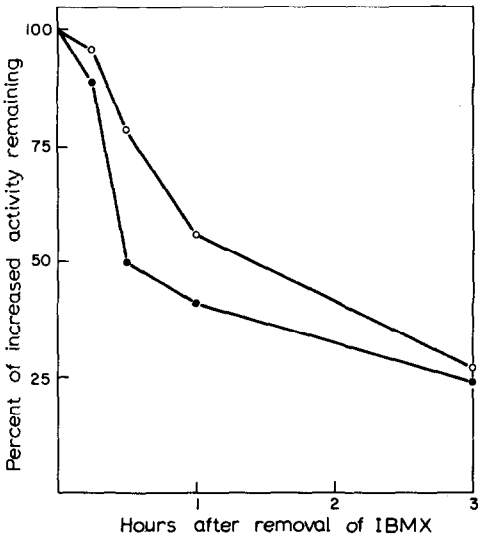

Fig. 2. Loss of 3-isobutyl-1-methylxanthine (IBMX) induced phosphodiesterase activity following removal of the inducer and incubation of cells with or without cycloheximide: Cells were treated with 250 μM 3-isobutyl-1-methylxanthine for 6 h; inducer was removed by washing the cells 4 times with fresh medium. Plates were divided into two batches: one with medium alone (○———○), the other with meidum plus 10 μg cycloheximide (●———●). At varous time intervals cells from both groups were harvested and assayed for phosphodiesterase activity with 5 μM cyclic AMP as substrate. Also activity of control cells (never exposed to the inducer) was determined at the various time points after washing cells to remove 3-isobutyl-1-methylxanthine. Percent of increased activity remaining at each time point (t_x) after removal of 3-isobutyl-1-methylxanthine is calculated as:

$$\frac{\left(\begin{matrix}\text{Phosphodiesterase activity at } t_x \\ \text{(cells exposed to the inducer)}\end{matrix}\right) - \left(\begin{matrix}\text{Phosphodiesterase activity} \\ \text{at } t_x \text{ (control cells)}\end{matrix}\right)}{\left(\begin{matrix}\text{Phosphodiesterase activity at } t_0 \\ \text{(cells exposed to the inducer)}\end{matrix}\right) - \left(\begin{matrix}\text{Phosphodiesterase activity} \\ \text{at } t_0 \text{ (control cells)}\end{matrix}\right)} \cdot 100\%$$

Each point represents duplicate determination of activity of 3 separate culture plates. Also, essentially similar results were obtained in two other separate experiments.

diesterase increased when WI-38 cells were exposed chronically to 0.2% serum at a very low density (Table III). The inducing ability of 3-isobutyl-1-methylxanthine under the above mentioned condition was completely absent.

Characterization of the induced enzyme in WI-38 cells

Distribution of activity in subcellular fractions. Various subcellular fractions were obtained by differential centrifugation of homogenates of control cells and of cells exposed to 3-isobutyl-1-methylxanthine for 6 h. The basal and the induced low K_m cyclic AMP phosphodiesterase activities showed similar distribution in the 1000—30 000 × *g* pellet (specific activity 0.4 times that of the whole homogenate) and in the 30 000 × *g* supernatant (specific activity 1.2 times that of the homogenate). The induced as well as the basal enzyme activity was found in about equal amounts in the pellet and soluble fractions.

Effect of cyclic GMP on the hydrolysis of cyclic AMP. When present at 10 μM cyclic GMP failed to compete with or inhibit degradation of cyclic AMP at 5 μM. Activity was slighly but significantly increased by cyclic GMP (+23% for control cells, +9% for 3-isobutyl-1-methylxanthine treated cells).

TABLE III

INFLUENCE OF CELL DENSITY AND SERUM ON CONTROL AND INDUCED PHOSPHODIESTERASE ACTIVITY IN WI-38 CELLS

Phosphodiesterase assays were carried out with 5 μM cyclic AMP as substrate and values represent means ± standard deviations as explained in Table I. For cell density experiments (A) WI-38 fibroblasts were first grown to various densities prior to treatment and harvesting. For experiments involving different serum concentrations (B) cells were plated initially at a very low density ($1 \cdot 10^4$ cells/cm^2) with fetal calf serum present at the usual (10%) concentration. On the next day the medium was replaced with fresh medium containing either 10% or 0.2% serum. Cells were then kept for 3 more days prior to treatment and harvesting.

	Serum concentration in cell culture medium (%)	Cell protein (μg/60 mm dish)	Phosphodiesterase activity (pmol cyclic AMP degraded/min per mg protein)	
			Control	Treated *
A	10	150—200	160 ± 40	363 ± 50
	10	300—400	250 ± 30	345 ± 35
	10	800—1000	150 ± 45	280 ± 80
	10	1200—1500	145 ± 20	245 ± 40
B	0.2	200—220	262 ± 36	245 ± 33
	10	630—660	100 ± 15	190 ± 15

* Cells were exposed to 250 μM 3-isobutyl-1-methylxanthine 6 h prior to harvesting.

Even when present at 100 μM, cyclic GMP only weakly inhibited (competed for) degradation of 5 μM cyclic AMP (—62% for control cells; —36% for treated cells).

Determination of the energy of activation of basal and 3-isobutyl-1-methylxanthine induced activity. Phosphodiesterase activity of control and treated cells was determined at 5 μM cyclic AMP substrate concentration with the temperature of assay incubation varied from 0—40°C. When log specific activity (pmol cyclic AMP degraded/min per mg protein) is plotted versus absolute temperature according to Arrhenius' equation in both instances, a linear relationship is obtained with a calculated energy of activation 5791 cal/mol.

Discussion

The present study provides evidence for the existence of more than one cyclic nucleotide phosphodiesterase in human lung fibroblasts and for the regulation of one of these phosphodiesterases by cyclic AMP. Thus, there appears to be one low K_m phosphodiesterase selective for cyclic AMP, another low K_m phosphodiesterase capable of degrading cyclic GMP and in addition, high K_m activity for both cyclic AMP and cyclic GMP, indicative of at least one additional enzyme. All these activities were increased with increase in cell density. However, only the low K_m cyclic AMP phosphodiesterase could be induced by agents which increased intracellular levels of cyclic AMP. A similar selective induction also occurred with 3-isobutyl-1-methylxanthine in SV-40 virus transformed fibroblasts. Although several of the agents used cause large increases in cyclic AMP levels in the fibroblasts [12,13], the 2—3 fold increase in the levels which is produced by 3-isobutyl-1-methylxanthine is apparently

sufficient for maximal induction. The relatively small effect of epinephrine on phosphodiesterase activity was surprising in view of its effect on cyclic AMP levels and warrants further investigation. One probable explanation might be that epinephrine produces an additional and negative effect on phosphodiesterase activity by a mechanism not related to cyclic AMP.

The induction of phosphodiesterase in the WI-38 as in other cultured cells [1,4,5,7] appeared to be associated with de novo synthesis of the enzyme. The half-life of the newly synthesized enzyme in human fibroblasts was about 30 min (Fig. 2) compared to 70—80 min in chick embryo fibroblasts [4]. In glioma, neuroblastoma, liver and L-cells in culture, induction by cyclic AMP and turnover of ornithine decarboxylase [14—16] are also relatively rapid. Although the induced phosphodiesterase in WI-38 cells may exhibit more rapid turnover than the non-induced enzyme, both enzymes appear to have similar biochemical properties.

In each cell type studied here, 3-isobutyl-1-methylxanthine induced only one phosphodiesterase, which was selective for cyclic AMP over cyclic GMP. In contrast to our results with the C-6 astrocytoma cells, Schwartz and Passonneau found that along with the low K_m enzyme, the high K_m cyclic AMP phosphodiesterase also increased [7]. The reason for these conflicting results with the C-6 cells is not known. Norepinephrine was found to increase activity of one of two species of cyclic AMP phosphodiesterase (separable by gel electrophoresis) in rat C-2A astrocytoma cells; however, K_m was not determined [6]. Agents or conditions which elevate intracellular cyclic AMP levels were reported to increase low K_m but not high K_m cyclic AMP phosphodiesterase in 3T3 and SV-40 transformed 3T3 cells [1] and in L-929 fibroblasts [5]. It was shown that exposure of chick embryo fibroblasts to agents which elevated cyclic AMP levels resulted in enhanced phosphodiesterase with cyclic AMP as substrate but not with cyclic GMP as substrate [4].

Removal of serum and increased cell density resulted both in increased adenylate cyclase activity of homogenates as well as increased cyclic AMP levels in several cell types [9,12,13,17]. In the lung fibroblasts there also appears to exist an overall correlation of "basal" cyclic AMP level and low K_m cyclic AMP phosphodiesterase. This is reflected not only in changes that occur with cell density changes but also due to removal of serum [12,13]. Thus, removal of serum may result in increased adenylate cyclase activity, then increased cyclic AMP levels, leading to increased phosphodiesterase activity. This induction process would thus in turn limit the extent to which basal cyclic AMP levels would be altered by changes in cell density or serum concentration. This sequence of events would account for the more striking effects of cell density on adenylate cyclase activity than on cyclic AMP levels in the human fibroblasts [12,13].

Acknowledgments

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