MARKED REDUCTION OF CYCLIC GMP PHOSPHODIESTERASE ACTIVITY IN VIRALLY TRANSFORMED MOUSE FIBROBLASTS

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Dibutyryl cAMP induced the accumulation of both cAMP phosphodiesterase and cGMP phosphodiesterase in normal mouse fibroblast (3T3) and transformed fibroblast (3T3-SV 40). The increase was larger with cAMP phosphodiesterase. Although both enzyme activities were reduced in the transformed cells, the reduction of cGMP phosphodiesterase was more pronounced. The differential increase of cAMP phosphodiesterase by B_2cAMP and the disparate reduction of cGMP phosphodiesterase in the transformed cell suggest that the two enzymes are regulated separately.

INTRODUCTION

Cyclic adenosine 3':5'-monophosphate (cAMP) plays an important role in the regulation of cell growth and differentiation. In cultured fibroblasts, the cellular level of cAMP appeared to be inversely related to the growth rate (1). Cyclic AMP content was high in normal fibroblasts but low in transformed cells; upon transformation the level dropped within seconds (2). Further, cAMP levels rose at confluency in contact-inhibited cells but not in transformed cells (3). Increasing the cellular level of cAMP decreased the saturation density of contact-inhibited cells and restored density-dependent inhibition of growth in some transformed cells (4). A monograph on the role of cyclic nucleotides on neoplastic growth has appeared (5).

The cellular level of cAMP is controlled by the rate of synthesis catalyzed by adenylate cyclase and the rate of degradation catalyzed by cAMP phosphodiesterase. Regulation of phosphodiesterase appears complex. The enzyme (or enzyme system) is known to exhibit complex kinetics and to have multiple forms (6,7), at least one of which requires a protein activator for maximal activity (8,9). The synthesis of enzyme and its

activator is regulated separately, probably at the genetic level (10).

A study of phosphodiesterase from cultured fibroblasts offers advantage over mammalian tissues in that the enzyme is derived from a homogeneous cell population and that its activity can be modulated by cellular levels of cAMP (11,12).

Results from this investigation show that cAMP and cGMP phosphodiesterase activities are present in mouse fibroblasts. Both enzyme activities are increased by dibutyryl cAMP (B_2cAMP); although both activities are lower in the transformed cells, the reduction of cGMP phosphodiesterase was more severe.

MATERIALS AND METHODS

Chemicals

[^{3}H]-cAMP (S.A. 27 Ci/m mole) and [^{3}H]-cGMP (S.A. 0.5 Ci/m mole) were obtained from Schwarz/Mann. They were purified by thin-layer chromatography on cellulose sheets. The purified samples were stored in small fractions at -90°. AG1-X2 (a styrene polymer), 200-400 mesh, was obtained from Bio-Rad or Sigma. IRP-58 (a phenolic polyamine), 200-400 mesh, was a gift of Rohm and Haas. The resins were washed with 0.5 M NaOH, glass-distilled water, 0.5 M HCl and then thoroughly with glass-distilled water until the solution was pH 5. The resins were used as a slurry, one part resin to two parts water (v/v).

Cultured Mouse Fibroblasts

Normal mouse fibroblast (3T3) and fibroblast transformed by a Simian virus (3T3-SV40), were obtained from American Type Culture Collection. The cells were plated in Falcon tissue culture dishes (100 x 200 mm) to a final density between 150,000 and 400,000 cells per plate, and were bathed in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. The medium contained penicillin (50 U/ml) and streptomycin (50 µg/ml). The cells were incubated at 37° in a humidified 5%-CO_2 atmosphere, and were harvested before confluency. They were washed in cold 40 mM Tris-HCl

(pH 8.0), scraped with a teflon policeman, and then resuspended in the same buffer.

Assay of Cyclic 3':5'-Nucleotide Phosphodiesterase

The harvested mouse fibroblasts, after having been quickly frozen and thawed twice, were homogenized in a glass tissue grinder. The crude homogenate was used immediately for separate assay of cAMP phosphodiesterase and cGMP phosphodiesterase. Phosphodiesterase was assayed with radioactive cAMP or cGMP using an anionic exchange resin essentially as described by Thompson and Appleman (13). The reaction mixture contained 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 1 μM [^{3}H]-cAMP or [^{3}H]-cGMP, and an appropriate amount of enzyme in a total volume of 100 μl. The reaction was initiated by the addition of substrate. After 10 min at 30°, the reaction was terminated by placing the tubes in a boiling water bath for 45 sec. The tubes were cooled to 30°, and 50 μl of snake venom *Crotalus atrox*, (1.0 mg/ml) as a source of 5'-nucleotidase was added to convert 5'-AMP to adenosine. After another 10 min, 1 ml of an anionic exchange resin was added to terminate the reaction, the tubes were centrifuged and the resin and denatured proteins were sedimented. A fraction of the supernatant fluid which contained [^{3}H]-adenosine or [^{3}H]-guanosine but not the [^{3}H]-cyclic nucleotide was counted in a liquid scintillation spectrometer.

As shown elsewhere, AG1-X2 binds 40% of adenosine and 80% of guanosine under these conditions (14). The batch use of this resin therefore underestimates cAMP phosphodiesterase and cGMP phosphodiesterase by 40% and 80%, respectively. On the other hand, IRP-58 binds 5% of adenosine and 10% of guanosine, and thus gives more accurate determination of phosphodiesterase activities (see Table III). Most of our assays were done with IRP-58. All determinations were done in duplicates or triplicates. The data given have not been corrected for the underestimation by these resins.

Determination of Protein

Protein was measured according to Lowry *et al.* (15) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Table I summarizes phosphodiesterase activities assayed with 1 μM cAMP (cAMP phosphodiesterase) and 1 μM cGMP (cGMP phosphodiesterase) in crude homogenates of 3T3 and 3T3-SV40 cells. In 3T3 cells the specific activities of cAMP phosphodiesterase and cGMP phosphodiesterase were 62 and 41, respectively; and in 3T3-SV40 cells the two activities were 47 and 4, respectively. Thus, in transformed fibroblasts both enzyme activities were significantly lower but the reduction was much more pronounced with cGMP phosphodiesterase. The differential decrease of cGMP phosphodiesterase activity in the transformed cells suggests that the two enzymes are regulated separately.

As mentioned earlier, at least one form of phosphodiesterase requires a protein activator for activity. Decreased phosphodiesterase activity may result from a diminished level of enzyme or its activator. We have shown that although the activity of cAMP phosphodiesterase may fluctuate under different experimental conditions in 3T3 and 3T3-SV40 cells that of the

Table I

Cyclic 3':5'-Nucleotide Phosphodiesterase

Activities in 3T3 and 3T3-SV40 Cells

Cells	cAMP Phosphodiesterase	cGMP Phosphodiesterase
	(p moles/mg protein/min)	
3T3	62 ± 2 (5)	41 ± 1 (4)
3T3-SV40	47 ± 2 (3)	4 ± 0.4 (3)

Mouse fibroblasts were cultured and phosphodiesterases were assayed as described under Materials and Methods. The values in parenthesis indicate the number of experiments. The data given are mean ± SEM.

activator remained essentially constant (10). This suggested that the reduction of phosphodiesterase activities in the transformed cells was due to a decrease of the enzymes and not the activator.

B_2cAMP or agents that elevate intracellular levels of cAMP increased cAMP phosphodiesterase activity in 3T3 cells (11), L cells (12), neuroblastoma cells (16), and C-6 glioma cells (17) presumably by enhancing the synthesis of new protein. Results summarized in Table II show that B_2cAMP increases both cAMP and cGMP phosphodiesterase activities, but the increase was larger with cAMP than with cGMP phosphodiesterase, in agreement with the notion that the two activities are regulated separately (18). The

Table II

Differential Effect of B_2cAMP on cAMP and cGMP Phosphodiesterase in 3T3 and 3T3-SV40 Cells

Cell	Treatment	cAMP Phosphodiesterase	cGMP Phosphodiesterase
		(relative activity)	
3T3	none	100	100
	B_2cAMP	191 ± 1	143 ± 1
	B_2cAMP + Act. D	115 ± 1	120 ± 2
3T3-SV40	none	100	100
	B_2cAMP	344 ± 3	164 ± 2
	B_2cAMP + Act. D	83 ± 2	107 ± 2

3T3 and 3T3-SV40 cells were grown essentially as described under Materials and Methods. About 48 hrs after plating, the growth medium was changed. The new medium contained 1 mM B_2cAMP, 1 mM theophylline and, where indicated, actinomycin D (1 μg/ml). In the control plates, the medium was changed but it did not contain these drugs. The cells were incubated for an additional 10 hrs. They were harvested and assayed for phosphodiesterases. The data given are mean ± SEM of 3 to 5 experiments.

elevation of both activities was blocked by actinomycin D, indicating that the increase of enzyme activity involved the synthesis of new messenger RNA. Similarly, dibutyryl cGMP and 1-methyl-3-isobutylxanthine, a potent inhibitor of phosphodiesterase (19), increased both enzyme activities, and the increase was larger with cAMP phosphodiesterase than with cGMP phosphodiesterase. However, the extent of enzyme induction by these two agents was less than that by B_2cAMP (data not shown).

The accumulation of both phosphodiesterases by B_2cAMP appears contrary to the finding of Russel and Pastan (18); they observed that in chick embryonic fibroblast B_2cAMP increased cAMP phosphodiesterase but not cGMP phosphodiesterase. They used an anionic exchange resin, Ag 1-X2, in their assay. We have found that the batch use of this resin underestimates cAMP phosphodiesterase by 40% and cGMP phosphodiesterase by 80%, according to the procedure described originally (14). Because of the low basal cGMP phosphodiesterase activity in the chick embryonic fibroblast (18), an increase of cGMP phosphodiesterase activity by B_2cAMP could have escaped detection by their method (see Table III). In the present studies, we used IRP-58, which measured both cAMP and cGMP phosphodiesterase activity more accurately. An experiment demonstrating this is presented in Table III. In this experiment, we determined phosphodiesterase activities in both normal and transformed mouse fibroblast with or without B_2cAMP in the growth medium. The data clearly show that AG1-X2, in comparison to IRP-58, gives apparently lower phosphodiesterase activities, and that AG1-X2 underestimates cGMP phosphodiesterase more than cAMP phosphodiesterase. When the basal activity of cGMP phosphodiesterase is low, as in 3T3-SV40 cells (last column in Table III), the effect of B_2cAMP would be likely overlooked. Thus, on the basis of the data obtained with AG1-X2, one may conclude erroneously that B_2cAMP induces the accumulation of cAMP phosphodiesterase but not cGMP phosphodiesterase.

In summary, our results show that B_2cAMP differentially increased cAMP

Table III

Comparison of cAMP Phosphodiesterase and cGMP Phosphodiesterase in 3T3 and 3T3-SV40 Cells Assayed with Different Anionic Exchange Resins

Cell	Treatment	cAMP Phosphodiesterase		cGMP Phosphodiesterase	
		IRP-58	AG1-X2	IRP-58	AG1-X2
		(p moles/mg protein/min)			
3T3	none	64	49	34	11
	B_2cAMP	120	86	50	9
3T3-SV40	none	46	31	3.5	1.2
	B_2cAMP	158	97	6.2	1.7

3T3 and 3T3-SV40 cells were cultured as described in the legend to Table II. Phosphodiesterases were assayed concurrently, in two sets, one with the batch use of IRP-58 and the other, AG1-X2. IRP-58 underestimated cAMP phosphodiesterase and cGMP phosphodiesterase by 5% and 10%, respectively, whereas AG1-X2 underestimated the two phosphodiesterases by 40% and 80%, respectively. These values were based on separate assays by paper chromatography, which gives accurate measurement of phosphodiesterase (14). The data given here are uncorrected values.

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phosphodiesterase in mouse fibroblast and that although both enzyme activities were lower in the transformed cells, the reduction was more severe with cGMP phosphodiesterase. The differential increase in cAMP phosphodiesterase by B_2cAMP and the disparate reduction in cGMP phosphodiesterase in 3T3-SV40 suggest that the two enzymes are regulated separately.

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