# SEPARATE GENETIC REGULATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE AND ITS PROTEIN ACTIVATOR IN CULTURED MOUSE FIBROBLASTS

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Dibutyryl cyclic AMP stimulated the synthesis of cyclic nucleotide phosphodiesterase (EC 3.1.4.17) but not its protein activator in normal mouse fibroblasts (3T3) and those virally transformed (3T3-SV 40). Stimulation appeared to be at the transcriptional level. These findings indicate that the increase of phosphodiesterase is not accompanied by its activator and that the two proteins are controlled by separate genes.

Increasing attention has been focused on cyclic nucleotide phosphodiesterase (EC 3.1.4.17), an enzyme or enzyme system that catalyzes the hydrolysis of cyclic AMP and thereby plays a key role in regulating its intracellular level. The mammalian enzyme is known to exhibit multiple forms (1-3), complex kinetics (4, 5) and a requirement for an endogenous protein activator for maximum activity (6). The activator, first discovered in bovine brain (7) and subsequently detected in many other tissues (8), has been purified to apparent homogeneity and extensively characterized in several tissues (9-14). Although it is specific for phosphodiesterase, it lacks tissue or species specificity (6).

Dibutyryl cyclic AMP ($B_2$cAMP) or agents that elevate intracellular levels of cAMP increased phosphodiesterase activity in 3T3 cells (15), L cells (16), neuroblastoma cells (17), and C-6 glioma cells (18) presumably by enhancing the synthesis of new protein. But the important question whether the elevated activity was due to increase of the enzyme or the activator or both has not been established. In this note, we determined separately the activities of phosphodiesterase and its activator in cultured mouse fibroblasts and show that the increase of enzyme activity results from

Table 1. Induced Synthesis of Phosphodiesterase by Dibutyryl Cyclic cAMP

| Expt. | Cells | Addition | Phosphodiesterase (pmoles/mg protein/min) | Activator (units/mg protein) |
|---|---|---|---|---|
| I | 3T3 (60 hrs) | none | 180 | 5.0 |
| | | $B_2$cAMP | 600 | 6.3 |
| II | 3T3-SV 40 (40 hrs) | none | 67 | 6.3 |
| | | $B_2$cAMP | 223 | 6.6 |
| III | 3T3-SV 40 (9 hrs) | none | 102 | 5.5 |
| | | $B_2$cAMP | 258 | 6.2 |
| | | $B_2$cAMP + Actinomycin D | 102 | 6.0 |
| | | $B_2$cAMP + Cycloheximide | 122 | 5.3 |

3T3 or 3T3-SV 40 cells were plated in Falcon tissue culture dishes (100 x 20 mm) to a final density between 150,000 and 400,000 cells per plate. Twenty-four hrs after plating, the medium was changed; the new medium contained $B_2$cAMP (1 mM), theophylline (1 mM) and actinomycin D (0.5 µg/ml) or cycloheximide (10 µg/ml) as shown in this table. In the control experiments, these drugs were omitted. The cells were then incubated further and were harvested before confluency at times indicated. They were washed in cold 40 mM Tris HCl (pH 8.0), scraped with a teflon policeman, and then resuspended in the same buffer. After having been frozen and thawed twice, they were homogenized in a glass tissue grinder. Crude homogenates were determined separately for cAMP phosphodiesterase and its activator. Phosphodiesterase was assayed with radioactive cAMP using an anionic exchange resin essentially as described previously (3). The reaction mixture contained 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 1 µM [$H^3$]-cAMP and an appropriate amount of enzyme in a total volume of 100 µl. This technique underestimated cAMP phosphodiesterase activity by about 40% (19) and the data presented in Table 1 had been corrected accordingly. To determine the activator, the tissue homogenate was boiled for 2 min to inactivate endogenous phosphodiesterase; the activator, which is heat stable (6), was then estimated by its ability to stimulate an activator-deficient phosphodiesterase prepared from bovine brain (20). One unit of activator is defined as that amount of protein to give half maximum stimulation of phosphodiesterase under standard conditions (6). Protein was determined according to Lowry *et al.* (21) with bovine serum albumin as a standard. The experiments described in Table 1 was repeated 3 times and the data were essentially identical.

an induced synthesis of the enzyme and not the activator, and that the two proteins are regulated separately at the genetic level.

3T3 and 3T3-SV 40 cells were cultured in Eagle's medium modified by Dulbecco with 10% fetal calf serum. The medium routinely contained 50 U/ml of penicillin and 50 µg/ml of streptomycin. All cells were incubated at 37°C in a humidified 5% $CO_2$ atmosphere, and were harvested before confluency.

The effect of $B_2$cAMP on the activities of phosphodiesterase and its activator in 3T3 and 3T3-SV 40 cells are shown in Table 1. In Expt. I, the enzymic activity of 3T3 cells in the presence of $B_2$cAMP was 3 times higher than in its absence. The activity of the activator, however, remained essentially the same. A comparable experiment, II, using 3T3-SV 40 cells, also showed an increase of phosphodiesterase activity without a significant change in the activator. Two possibilities may account for the differential enhancement of phosphodiesterase activity. One is that $B_2$cAMP induces the synthesis of phosphodiesterase without affecting that of the activator. The other is that $B_2$cAMP stimulates the synthesis of both proteins, but that $B_2$cAMP also accelerates the turnover of the activator such that its net level remains the same. To exclude the second possibility, we examined the effect of $B_2$cAMP on the activities of the two proteins in the presence of actinomycin D or cycloheximide, inhibitors of protein synthesis at the level of transcription or translation, respectively. Expt. III shows that both agents suppressed the stimulated activity of phosphodiesterase, indicating that the increase of enzymic activity required the synthesis of new messenger RNA. If $B_2$cAMP accelerates the turnover of the activator, its activity should be decreased in the presence of the inhibitors. The fact that neither of these drugs change the level of the activator (Expt. III) would argue against the notion that $B_2$cAMP augments its turnover.

Table 1 further shows that the activity of the activator remained relatively constant in spite of the fact that the activity of the enzyme varied substantially from one experiment to another and that the cells were harvested at different times after plating. In other experiments in which 3T3 and 3T3-SV 40 cells were harvested at the same time, the level of the activator was again in the same range as in Table 1, whereas that of phosphodiesterase was invariably lower in the transformed cells (data not shown).

We conclude from these data that $B_2$cAMP stimulates the synthesis of

phosphodiesterase but not of the activator and that the two proteins are regulated separately, presumably by different genes.

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