INDUCTION OF ALKALINE PHOSPHATASE BY CYCLIC AMP OR ITS DIBUTYRYL DERIVATIVE IN A HYBRID LINE BETWEEN MOUSE AND CHINESE HAMSTER IN CULTURE

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SUMMARY: Cyclic AMP (c-AMP) and its derivative, dibutyryl-cyclic AMP (DB c-AMP) induced the activity of alkaline phosphatase (ALP) in a hybrid line. Theophylline was also effective and greatly potentiated the action of c-AMP when given along with the nucleotide. This effect was attributable neither to a direct activation of the enzyme nor to the formation of activators in the cells grown in DB c-AMP. In the presence of actinomycin S_3 or cycloheximide, the induction by DB c-AMP did not occur or was reduced very much. It is therefore suggested that c-AMP and DB c-AMP induce alkaline phosphatase activity through a new synthesis of both RNA and protein in the cells.

In some cultured mammalian cells, alkaline phosphatases (ALP) have been reported to be induced by prednisolone (1), substrates such as phenylphosphate or β-glycerophosphate (2), hyperosmolarity (3), and low temperature (4). Recently, we reported a pronounced induction of ALP by 5-bromodeoxyuridine (BUdR) in the hybrid line, B-6, derived from hybridization of mouse and Chinese hamster cell lines in vitro (5). This line has the inheritable ability to produce hyaluronic acid (6, 7), and the synthesis is inhibited reversibly by BUdR treatment (8).

During the course of studies on the mechanisms of the induction of alkaline phosphatase and inhibition of the mucopolysaccharide synthesis by BUdR, a striking rise in ALP activity was observed in response to cyclic AMP (c-AMP) or its dibutyryl derivative (DB c-AMP) added. The present paper describes the new phenomenon and some

results of experiments carried out for elucidation of this mechanism.

MATERIALS AND METHODS

The hybrid line (B-6) was grown in suspension cultures in a modified Eagle's medium (Nissui Seiyaku, Tokyo), supplemented with 5% calf serum and 0.1% Bacto-Peptone (Difco, Detroit) as described previously (6-8).

The methods for treatment of B-6 cells with chemicals and preparation of cell-free extracts for enzyme assay were the same as those cited in the preceding paper (5), except for the use of 50-mm Toyoshima plastic dishes (Toyoshima Seisakusho, Tokyo) containing 5 ml of the above medium and test compound in place of glass dishes. Alkaline and acid phosphatase activity of cell-free sonicates was measured by the method of Lowry (9). Specific activity was expressed as nmoles of p-nitrophenol released in 1 hr per mg protein. Protein was determined by the method of Oyama and Eagle (10) using bovine serum albumin as a standard.

The medium containing c-AMP or DB c-AMP was prepared by adding each drug to the medium and then sterilizing it with a Millipore filter just before use. c-AMP was purchased from Daiichi Pure Chem., Tokyo, and DB c-AMP from Boehringer Mannheim Japan, Tokyo. Actinomycin S_3, which inhibits RNA synthesis similarly to actinomycin D, was kindly supplied by Daiichi Pure Chem. Cycloheximide was obtained from Kaken Kagaku, Tokyo.

RESULTS

Table 1 indicates the alkaline phosphatase activity in the cells treated for 3 days with varying concentrations of c-AMP or DB c-AMP in the presence and absence of theophylline. c-AMP added in 0.4 mg/ml induced the activity to some extent, and cultures treated with 1 mg/ml had 2.8 times higher activity than the control. DB c-AMP was much more effective than the natural nucleotide. A marked induc-

Additions	Final concentration	Alkaline phosphatase	Acid phosphatase
	(mg/ml)	(n moles/hr/mg protein)	
None	——	55.3	1220
c-AMP	0.4	98.7	1160
c-AMP	1.0	196	1160
Theophylline	0.18	137	1060
c-AMP + Theophylline	0.4 + 0.18	413	1280
c-AMP + Theophylline	1.0 + 0.18	645	1280
None	——	48.8	1340
DB c-AMP	0.05	72.8	1480
"	0.25	186	1570
"	0.50	680	1720
"	1.0	3650	1920
"	2.5	14400	3060

Table 1. Effect of c-AMP and DB c-AMP on alkaline and acid phosphatase activity.
2.5×10^5 viable cells were incubated for 3 days in various media in the presence of c-AMP, DB c-AMP or theophylline at the concentrations indicated.

tion was observed in parallel with the concentration of the derivative added. At the maximal concentration (2.5 mg/ml) examined, the activity was as much as 300-fold of the level of controls.

A lesser rise in the activity was also observed when the cells were grown in the medium containing theophylline (0.18 mg/ml), and the simultaneous addition of theophylline and c-AMP gave a higher level of activity than the additive value of activities due to each reagent employed separately. In contrast, acid phosphatase showed no significant changes in activity after the cells were cultured

with these chemicals except that DB c-AMP at higher concentrations produced some increase in the phosphatase level.

Increase in ALP was not observed 24 hr after addition of DB c-AMP (0.5 mg/ml), and thereafter a marked elevation proceeded linearly without showing any plateau even after 72 hr. Subsequently, following transfer of the induced cells to the drug-free medium, the high ALP level returned to the original in 3 days.

c-AMP is known to activate phosphorylase b kinase kinase (11), protein kinase (12), etc. as an allosteric effector. It was therefore expected that the same mechanism might be involved in the induction of alkaline phosphatase by c-AMP or DB c-AMP. As seen in Table 2, however, this possibility was excluded since the addition of these

Cell-free extracts	Additions	Alkaline phosphatase
		(nmoles/hr/mg protein)
I	——	35.6
II	——	324
I + II	——	189 (180)
I	Distilled water, 0.1 ml	33.6
"	c-AMP, 0.4 mg/ml	31.2
"	c-AMP, 1 mg/ml	36.0
"	DB c-AMP, 0.5 mg/ml	33.6
"	DB c-AMP, 1 mg/ml	28.8

Table 2. Exclusion of the possibility for c-AMP or DB c-AMP to activate directly alkaline phosphatase or the presence of activators in the treated cells.
Cell-free extracts, I and II, were prepared from the cultures grown for 3 days in the absence and presence of DB c-AMP at 0.5 mg/ml. To check the activation of ALP by the nucleotides, they were dissolved in distilled water, 0.1 ml of the solutions was added to the reaction mixtures at the final concentrations indicated, and the assay was run in the usual manner. On the other hand, to test the possibility of presence of activators in the treated cells, two cell-free extracts were mixed together in the same volume and the assay was carried out. The value in parenthesis is the arithmetical mean of both activities.

compounds to the enzyme solution prepared from control cultures did not result in the rise of activity. Besides, the activity of mixtures of cell-free extracts was equal to the arithmetical mean of both activities, suggesting that the elevation in ALP levels is neither due to activators in the cultures treated with both nucleotides nor to inhibitors in control cultures (Table 2).

The effect of actinomycin S_3 and cycloheximide on induction is represented in Table 3. At the concentrations used here, actinomycin S_3 inhibited RNA synthesis of the B-6 cells by more than 95%, while cycloheximide inhibited protein synthesis by more than 93% after one generation (16 hr). There was no change in the normal basal level of ALP activity 34 hr (two generations) after the addition of either of the antibiotics. In contrast, a 6.9-fold increase in activity with DB c-AMP (1 mg/ml) was completely prevented by actinomycin S_3. In the presence of cycloheximide, on the other hand,

Additions	Alkaline phosphatase	Acid phosphatase
	(n moles/hr/mg protein)	
None	43.0	1240
Actinomycin S_3	41.5	1170
DB c-AMP	689	1260
Actinomycin S_3 + DB c-AMP	57.5	1150
None	38.0	1340
Cycloheximide	41.5	1150
DB c-AMP	698	1400
Cycloheximide + DB c-AMP	113	1140

Table 3. Effect of actinomycin S_3 (0.25 µg/ml) and cycloheximide (1 µg/ml) on the induction of alkaline phosphatase by DB c-AMP (1 mg/ml).
10^6 viable cells were plated on dishes containing 5 ml of the medium with DB c-AMP and, at the same time, actinomycin S_3 or cycloheximide was added to them, followed by cultivation for 34 hr (two cell generations).

the induced activity was reduced to one-sixth, though this activity was still 3 times higher than the basal level. These data indicate that a new synthesis of both RNA and protein is required for c-AMP or DB c-AMP to induce alkaline phosphatase activity.

DISCUSSION

The present data show the induction of alkaline phosphatase activity by c-AMP and DB c-AMP in the B-6 line. Similar changes in the enzyme have also been found to occur in the same cells grown in BUdR (5). By comparing a variety of cells in culture with respect to the inducibility by DB c-AMP and BUdR, we have found that not all of them respond. These results will be reported elsewhere. On the other hand, with the B-6 cells, none of the factors described in the Introduction was effective, which had been reported to elevate ALP activity in some cultured cells (1-4).

Several experiments for elucidating the mechanism of induction by c-AMP and its derivative indicate that the response depends neither upon the activation of enzyme by an allosteric effect nor upon the formation of activators in the treated cells. Rather, there is some evidence for increased synthesis of enzyme protein, since actinomycin S_3 and cycloheximide block the induction completely or to a great degree.

Recently, Chader (13) reported that glutamine synthetase in chick retina cells *in vitro* is induced by c-AMP or DB c-AMP and that *de novo* enzyme synthesis is involved in the reaction because perfect blocking the induction was observed in the presence of both antibiotics used above. In contrast, DB c-AMP has been found to act at some posttranscriptional level to induce tyrosine aminotransferase in rat hepatoma cultures (14). Furthermore, Hsie and Puck (15) and Johnson et al. (16) observed drastic changes or transformation in morphology of some cultured cell lines, and the latter authors found no require-

ment of RNA synthesis for the response. c-AMP not only plays a well-defined role as a "second messenger" in the action of many hormones (17), but also has many effects in different biological systems, as well as in cultured mammalian cells. The mechanisms of these effects therefore will differ greatly in each system. More detailed studies in our system are now in progress.

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