ENHANCEMENT OF ALKALINE PHOSPHATASE ACTIVITY IN MOUSE L-FIBROBLASTS BY CYCLIC AMP AND CHOLERAGEN

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The L_A subline of mouse L-fibroblasts shows a high basal activity of alkaline phosphatase which significantly increases 24—48 h after the administration of compounds enhancing the intracellular level of cyclic AMP. Cyclic AMP (5 mM) in combination with theophylline (1 mM) brings about a two-fold increase of the activity of alkaline phosphatase. Far stronger effects can be observed after the application of dibutyryl cyclic AMP itself (2 mM) or of choleragen (5 μg per $5 . 10^6$ of cells). The combination of these compounds and theophylline caused an approximately 10-fold increase of activity of alkaline phosphatase in 48 h. Adenosine (1 mM), one of the compounds inhibiting the choleragen-stimulated activity of adenylate cyclase *in vitro*, was used for the inhibition of alkaline phosphatase. This nucleoside blocked the stimulating effect of choleragen by 70—80%. The relation between the level of cyclic AMP, the activity of alkaline phosphatase, and the rate of cell growth is discussed.

During extensive studies on the metabolic characteristics of mouse L-fibroblasts we were able to obtain subline L_A which shows a high basal activity of alkaline phosphatase (EC 3.1.3.1). Since the relation between the rate of growth of certain types of cells and the activity of alkaline phosphatase has been reported[1,2], we decided to investigate the activity of this enzyme in L_A cultures after the administration of compounds which alter the intracellular level of cyclic AMP. This nucleotide is known to modify the rate of growth and the differentiation of cells[3-6] in a number of tissue cultures.

In this study we used as model system with enhanced level of cyclic AMP a tissue culture which was exposed to purified cholera toxin, choleragen, known to have marked stimulating effects on the activity of adenylate cyclase[7] in mouse L_A fibroblasts. The enhancement of the activity of alkaline phosphatase after the application of this toxin was compared with the effect of other compounds which also increase the intracellular level of cyclic AMP. Of the compounds which inhibited the choleragen-stimulated activity of adenylate cyclase *in vitro*, adenosine was used to inhibit the activation of alkaline phosphatase.

EXPERIMENTAL

Chemicals. The TM Sevac III (Epl) medium for the cultivation of cells was prepared in the Institute for Sera and Vaccines, Prague. Highly purified cholera exo-enterotoxin, choleragen (No 0572), was prepared by Dr R. A. Finkelstein[8] under contract for the National Institute of Allergy and Infectious Diseases. Prostaglandin E_1 was a gift from Dr E. Pike, Upjohn Co., Kalamazoo, Michigan. Adenosine 5'-triphosphate (trisodium salt), 2-phosphoenol pyruvate (tricyclohexylammonium salt), rabbit muscle pyruvate kinase (300 I. U. per mg of protein), rabbit muscle myokinase (660 I. U. per mg of protein), and adenosine deaminase (10 mg per ml) were products of Calbiochem, Lucerne. Theophylline, adenosine, and cyclic AMP were from Lachema, Brno, dibutyryl cyclic AMP and adenosine 5'-diphosphate from Boehringer, Mannheim. Neutral alumina for chromatographic adsorption analysis according to Brockmann (II) was purchased from Reanal, Budapest. Adenosine 5'-triphosphate-[α ^{32}P], sodium salt, (500 to 10000 mCi/mmol) was supplied by the Radiochemical Centre, Amersham.

Cultivation of cells. The L_A subline of mouse L-fibroblasts was grown as monolayer cultures in TM Sevac III (Epl) medium without the addition of serum. The concentration of antibiotics added was 100 μg of penicillin and 100 μg of streptomycin per 1 ml of medium. When passaged the cells were released from the glass by 0·2% trypsin solution in isotonic phosphate buffer. Cultures 3 days after the last trypsinization (*i.e.* in the logarithmic phase of growth) were used in all experiments. The density of cells, at the time when the modifying compounds were added, was $5 \cdot 10^6$ cells per culture bottle containing 20 ml of medium. The individual compounds added were dissolved in fresh, sterile medium and the solutions allowed to stay over the tissue culture until the time of treatment (exposure 6—48 h). An exception was made with choleragen which was added to the tissue culture without the medium (5 μg/0·5 ml of water). After 5 min exposition the toxin was decanted off and replaced by fresh medium. The cells bound for the obtaining of enzyme preparations were set free from the glass by a rubber spatula.

The activity of adenylate cyclase was measured in cell homogenates by the method and procedure described elsewhere[7]. The activity of alkaline phosphatase was measured in deoxycholate lysates of cells[9]. The washed cells obtained by centrifugation were suspended in 1% sodium deoxycholate ($2 \cdot 10^6$ cells per ml); after 20 min of stirring at room temperature the sample was diluted with an equal volume of 0·9% NaCl. The measurement of the activity of alkaline phosphatase was made in a glycine buffer, pH 10·0, containing 1 mM-$MgCl_2$ and 2,4-dinitrophenyl phosphate (10 mM) as substrate. The quantity of 2,4-dinitrophenol liberated was determined spectrophotometrically at 410 nm. The phosphatase activity was expressed as μg of 2,4-dinitrophenol liberated per mg of protein in 1 h. The protein content was determined by the method of Lowry and coworkers[10]. All determinations were made in triplicate. Unless the statistical evaluation is described, the results given are those of representative experiments.

RESULTS

In the first part of this study we examined the activity of adenylate cyclase in homogenates of mouse L-fibroblasts. The addition of optimum concentrations of choleragen (5 μg per $5 \cdot 10^6$ of cells) caused a significant enhancement of the basal enzyme activity 6—24 h after the exposure of cells to choleragen; the effect was maximum essentially after 12 h (Fig. 1). The enzyme were prepared from cells detached from the glass by a rubber spatula.

The stimulating effect of prostaglandin E_1(PGE_1) and of sodium fluoride is significantly decreased with adenylate cyclase preparations obtained from cultures which have been exposed to the action of choleragen for 24 h. Whereas both compounds bring about a multifold enhancement of the basal enzyme activity in controls, the stimulating effect of PGE_1 and sodium fluoride on preparations exposed to choleragen manifests itself by a slight increase only (20–30%). The results are summarized in Fig. 2.

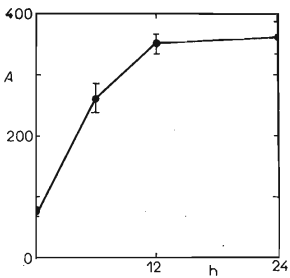

FIG. 1

Effect of Choleragen on Adenylate Cyclase in Mouse L_A Fibroblasts

The cultures after trypsinization were grown for 3 days ($4-5 \cdot 10^6$ of cells). After the medium had been decanted off, a solution of 5 μg of choleragen in 0·5 ml of water was poured on the cultures. The toxin was removed 5 min thereafter, a fresh medium was added, and the cultivation was continued for 6–48 h. The cells were mechanically released from the glass and homogenized in an all-glass homogenizer. The basal activity of adenylate cyclase was determined with ATP-[α ^{32}P]. The cyclic AMP formed was isolated and expressed in pmol per mg of protein per 10 min incubation at 37°C. The values given are means of 5 independent experiments ± SE.

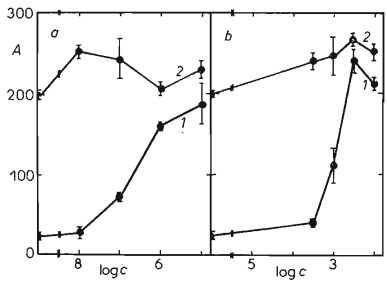

FIG. 2

Comparison of Stimulating Effect of Prostaglandin E_1 (*a*) and Sodium Fluoride (*b*) on Activity of Adenylate Cyclase in Control L_A Cells and Cultures Exposed to Action of Choleragen

1 Control cultures, *2* cultures exposed to choleragen for 24 h (5 μg per $5 \cdot 10^6$ of cells). The values given are means of 5 independent experiments ± SE.

In subsequent experiments we made a search for compounds which would antagonize the increased activity of adenylate cyclase in preparations of cells exposed to the action of choleragen. In addition to the inhibitory effect of prostaglandin antagonists[11] (7-oxa-13-prostynoic acid and polyphloretin phosphate) we observed that strong inhibitory effects on the choleragen-stimulated activity of adenylate cyclase had shown 5'-AMP and adenosine (Fig. 3). Both these compounds participate[12] most likely on physiological regulation of intracellular concentration of cyclic AMP. The effect of adenosine can be quenched by the addition of adenosine deaminase (1 μg/1 ml), which converts this nucleoside into inosine.

In the second part of our study we examined the activity of alkaline phosphatase in the L_A subline of mouse fibroblasts after the application of compounds which increase the intracellular level of cyclic AMP. Fig. 4 shows the time profile of increase of activity of alkaline phosphatase after an exposure of the tissue culture to the action of choleragen; the results of several independent experiments are presented. Unlike with adenylate cyclase where maximum activity of the enzyme was obtained already 12 h after the addition of the toxin, a significant enhancement of the activity of alkaline phosphatase was observed only after 24 h and the activity kept increasing till the 48th hour (longest interval of investigation). This finding helped us to determine the time period necessary for the examination of the activity of alkaline phosphatase in subsequent experiments.

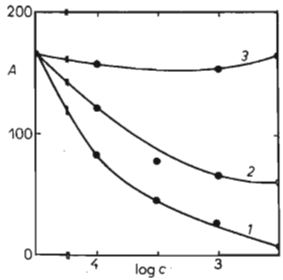

FIG. 3

Inhibitory Effect of 5'-AMP (*1*), Adenosine (*2*), and of its Combination with Adenosine Deaminase (*3*) on Activity of Adenylate Cyclase in L_A Cells Exposed to Action of Choleragen.

The experimental conditions are those described in the legend to Fig. 1; the cell cultures were exposed to the action of choleragen for 24 h.

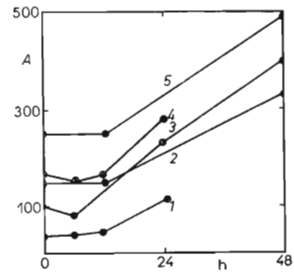

FIG. 4

Stimulating Effect of Choleragen on Activity of Alkaline Phosphatase in L_A Cells

The cells cultivated for 3 days after the last trypsinization ($4-5 \cdot 10^6$ of cells) were exposed for 5 min to the action of the toxin (5 μg per $5 \cdot 10^6$ of cells), then the toxin was removed, and a fresh culture medium was added. The values given are means of five independent experiments (*1–5*).

We endeavored to verify the assumption that the condition of the increase of activity of alkaline phosphatase after the exposure of L_A fibroblasts to choleragen is related to an increase of the intracellular level of cyclic AMP. We chose an indirect procedure, *i.e.* the cell cultures were exposed to the action of cyclic AMP alone, of its dibutyryl

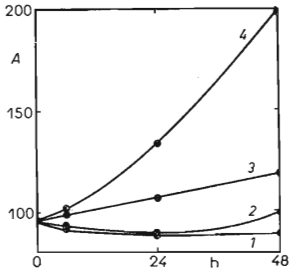

FIG. 5

Effect of Cyclic AMP-theophylline and their Combination on Activity of Alkaline Phosphatase in L_A-Cells

Activity of alkaline phosphatase in *1* control cultures, *2* after treatment with 1 mM theophylline, *3* after 5 mM cyclic AMP and *4* after simultaneous treatment with 5 mM cyclic AMP and 1 mM theophylline.

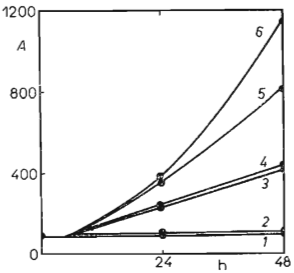

FIG. 6

Effect of Dibutyryl Cyclic AMP, Choleragen, and their Combination with Theophylline on Activity of Alkaline Phosphatase in L_A Fibroblasts

The experimental conditions are those described in the legends to Fig. 4 and 5. Activity of alkaline phosphatase: *1* in control cultures; *2* after treatment with 1 mM theophylline; *3* after choleragen (5 μg per $5 \cdot 10^6$ of cells); *4* 2 mM dibutyryl cyclic AMP; *5* after choleragen (5 μg per $5 \cdot 10^6$ of cells) and theophylline; and *6* after simultaneous treatment with 2 mM dibutyryl cyclic AMP and 1 mM theophylline.

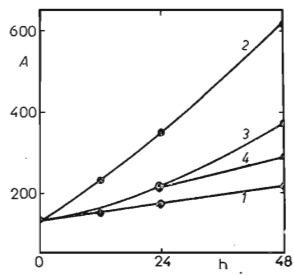

FIG. 7

Inhibitory Effect of Adenosine on Choleragen-Simulated Activity of Alkaline Phosphatase in L_A Fibroblasts

The experimental conditions are those described in the legend to Fig. 6. Activity of alkaline phosphatase: *1* in control cultures; *2* in cultures treated with choleragen (5 μg per $5 \cdot 10^6$ of cells); *3* a combination of choleragen and one adenosine dose (1 mM); and *4* a combination of choleragen and two adenosine doses (1 mM) at 0 and 24 h of incubation.

derivative, and of their combination with theophylline. The combination of cyclic AMP (5 mM) with theophylline (1 mM) brought about a two-fold increase of control values in 48 h although none of the compounds used possessed by itself a marked stimulating effect (Fig. 5). Dibutyryl cyclic AMP (2 mM) was a far more effective stimulator of the activity of alkaline phosphatase since it caused, similarly to choleragen, a 4-fold increase of the basal values in 48 h. The addition of theophylline to dibutyryl cyclic AMP or choleragen led to an additional enhancement of the enzyme activity which increased to about the 10-fold of the basal values (Fig. 6).

Adenosine, which unlike 5'-AMP penetrates into the cells, was used in this study as an inhibitor of the activation of alkaline phosphatase. Since adenosine is degraded by adenosine deaminase which removes its inhibitory effects we compared the antagonistic effect of single-dose and repeated administration of this nucleoside on the effect of choleragen. As shown in Fig. 7, adenosine (1 mM) strongly inhibited the enhancement of the activity of alkaline phosphatase after the application of choleragen during the first 24 h. Unless this inhibitory effect is to stop, another dose of adenosine must be added at the 24 h incubation. Repeated application of adenosine antagonized the stimulatory effect of choleragen on the activity of alkaline phosphatase by 70–80%.

DISCUSSION

Cyclic nucleotides are to date believed to play two biologically important roles, namely in the regulation of growth rate[13] and in the process of cell differentiation[14]. The changes in the intracellular levels of cyclic AMP and cyclic GMP and their respective ratio can markedly modify the growth rate of many types of mammalian cells[15] and are one of the factors which can affect the contact inhibition of cell growth[16]. The mechanism by which cyclic nucleotides alter the growth rate has not been explained satisfactorily. Unclear also remains the part of alkaline phosphatase and the nature of its relation to the rate of cell growth as presented in literature[1,2].

Mouse L-fibroblasts represent a heteroploid cell line which can permanently be grown *in vitro*. In this study we tried to find out whether the activity of alkaline phosphatase in the L_A subline investigated can be enhanced *via* an increase of the intracellular level of cyclic AMP. The level of this nucleotide was changed toward higher values by the action of choleragen, by the administration of exogeneous cyclic AMP or dibutyryl cyclic AMP, and lastly by the addition of theophylline to these compounds. The rise of the activity of alkaline phosphatase induced by choleragen markedly decrease by the application of adenosine.

In this study we found a positive correlation between increased activity of adenylate cyclase and alkaline phosphatase after the application of choleragen. We assumed that, the measurement of the activity of adenylate cyclase was a more suitable criterion than the determination of intracellular level of cyclic AMP which rapidly increases and immediately decreases[17], *e.g.* after hormonal stimulation; therefore no good correl-

ation can be expected with changes which undergo a maximum within 48 h. The fact that exogenous cyclic AMP showed a slight stimulating effect only on the activity of alkaline phosphatase can be ascribed to its limited penetration into the cells[18]. This nucleotide can be also metabolized to ineffective products or adenosine which has an inhibitory effect on the stimulation of adenylate cyclase[12] (Fig. 3). Dibutyryl cyclic AMP ,which easily penetrates into cells and is degraded at a slower rate, showed a marked stimulating effect on the activity of the enzyme investigated (Fig. 6).

From the data presented here we conclude that the activity of alkaline phosphatase in the L_A subline of mouse fibroblasts grown *in vitro* is influenced by the intracellular concentration of cyclic AMP, even though this interference with this process can shift the level of this nucleotide beyond the limit of physiological values. It remains to be shown in forthcoming studies whether we are dealing with the induction of the synthesis of alkaline phosphatase as reported elsewhere[19,20] or whether we merely observe a change in the activity of the enzyme due to its activation. It must be also determined whether in the cell population examined exists a relation between the growth rate and phosphatase activity and whether this relation is a causal one; otherwise the increase of the activity of alkaline phosphatase would be of coincidental character, conditioned on the increase of the intracellular level of cyclic AMP.

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