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'Superactivation' of alkaline phosphatase activity by cycloheximide in rat hepatoma cell cultures

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We have found that rat hepatoma cells (R-Y121B) retain alkaline phosphatase activity, and that this enzyme activity is increased by cycloheximide. Actinomycin D also increased the enzyme activity. This increase due to actinomycin D was partially inhibited by cycloheximide. The characteristics of alkaline phosphatase of the cells treated or untreated with cycloheximide or actinomycin D were similar to each other; they were heat labile and the enzyme reaction was strongly inhibited by L-homoarginine, but weakly by L-phenylalanine. The increase in alkaline phosphatase activity with cycloheximide has been termed a 'superactivation' of alkaline phosphatase.

Alkaline phosphatase (EC 3.1.3.1) is retained in many cell lines (Cox & Pontecorvo 1961; Cox & MacLeod 1962; Maio & de Carli 1962; Koyama & Ono 1971; Nose et al 1973; Mizuno et al 1983). Although its specific activity is increased by actinomycin D in-vivo (Moog 1964, 1965; Grey & Moog 1966; Moog & Grey 1968), actinomycin D decreases the total amount of proteins in organs. So it is uncertain whether or not the drug increases total enzyme activity; if the degradation rate of other proteins in the presence of actinomycin D is faster than that of alkaline phosphatase, the specific activity of the enzyme increases without enzyme synthesis. Using cultured cells to assess the effect of actinomycin D or cycloheximide on the regulation of enzyme activity, we can estimate the total amount of enzyme activity. In the present study, we show that rat hepatoma cells (R-Y121B) (Niwa et al 1980), derived from Reuer hepatoma cells (H4-II-E) (Pitot et al 1964), have alkaline phosphatase activity that is enhanced by cycloheximide as well as by actinomycin D.

Materials and methods

Cycloheximide was purchased from Sigma Chemical Co. (St Louis, Mo., USA), Wako Pure Chemical Industries, Ltd (Osaka, Japan) and Nakarai Chemicals Ltd (Kyoto, Japan). Actinomycin D was purchased from Calbiochem (San Diego, Calif., USA). Puromycin dihydrochloride was obtained from ICN NBC Laboratories Inc. (Cleveland, Ohio, USA). L-Homoarginine HCl and L-phenylalanine were obtained from Nakarai Chemicals Ltd and Ajinomoto Co., Ltd (Tokyo, Japan), respectively.

Rat hepatoma cells (R-Y121B) maintained in glass culture flasks were transfered to 35-mm diameter plastic culture dishes (Corning, NY, USA), containing a modified Eagle's minimum essential medium containing 0·5 or 5% calf serum and in which glutamine was replaced with glutamic acid (Sorimachi et al 1980) and cells were cultured in an atmosphere of 5% CO_2 and 95% air at 37 °C (Sorimachi et al 1980). An inoculation of $\approx 5 \times 10^5$ cells almost reached confluence after one week. Then, the cells were treated with cycloheximide or actinomycin D. Cells were harvested, centrifuged at 800*g* for 5 min then homogenized in a Teflon-glass Potter-Elvehjem type homogenizer with 0·2–0·5 ml of H_2O at 0 °C (Sorimachi et al 1980).

Alkaline phosphatase activity was measured by the method of Kind & King (1954) modified according to Watanabe et al (1967). The substrate solution was 0·05 M carbonate-bicarbonate buffer, pH 10·15, with 4·2 mM disodium phenylphosphate and 2·2 mM 4-aminoantipyrine. Homogenate (50 µl) was mixed with 1 ml substrate solution and incubated at 37 °C for 10 min. To measure the amount of liberated phenol from disodium phenylphosphate, 5·8 mM potassium ferricyanide in 0·21 M boric acid (1 ml) was added to the reaction mixture, and increase in absorbance at 500 nm was measured. As reference, 50 µl of H_2O was used instead of homogenate. The amount of cellular protein was estimated by the method of Lowry et al (1951) with bovine serum albumin as the standard.

Results

When R-Y121B cells were incubated with either cycloheximide or actinomycin D (5 µg ml^{-1}), the specific activity of alkaline phosphatase increased during 5 h of preincubation at 37 °C (Fig. 1, upper panel). A large increase in the specific activity was found between 10 and 22 h of preincubation in both cases, more than 95% of the cells dying during 22 h of incubation at 37 °C. In other experiments, the increase in the specific activity of alkaline phosphatase was small between 24 and 48 h of incubation in the presence of either drug, both of which decreased the amount of total cellular protein (Fig. 1, middle panel). When the total amount of cellular protein decreased, the specific activity of enzymes eventually increased, even though the total enzyme activity was constant, therefore, the total enzyme activity was estimated (Fig. 1, lower panel). Even if the enzyme activity is expressed as the total, the present results show that both cycloheximide and actinomycin D significantly increased alkaline phosphatase activity in R-Y121B cells. When cells were incubated with these drugs at 4 °C, an increase in enzyme activity was not observed.

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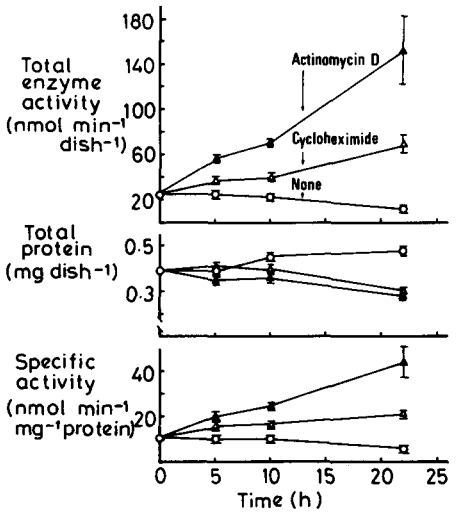

FIG. 1. Time courses of specific and total activity of alkaline phosphatase and total amount of cellular proteins in R-Y121B cells in the presence or absence of cycloheximide or actinomycin D (5 μg ml^{-1}). The value given is the mean ±s.d. of the values for three culture dishes.

Table 1. Effects of cycloheximide, actinomycin D or their combination on the total enzyme activity and the total amount of cellular protein in R-Y121B cells.

Cycloheximide	Actinomycin D 0	0·5 μg ml^{-1}	5·0 μg ml^{-1}
0	29·3 ± 3·4 (100)	129 ± 4 (67)	228 ± 6 (64)
0·5 μg ml^{-1}	39·1 ± 1·4 (78)	86·8 ± 3·8 (60)	136 ± 2 (64)
5·0 μg ml^{-1}	59·1 ± 2·8 (64)	79·7 ± 3·3 (58)	119 ± 1 (59)

Cells were treated with the drug(s) for 24 h at 37 °C. The total enzyme activity is expressed as nmol/10 min per dish. The values in parentheses are percent of the total amount of cellular proteins per dish of fresh cells that were not treated with the drug. The value given is the mean ±s.d. of the values for three or six culture dishes.

The increase in enzyme activity was investigated with different drug concentrations and combinations. The increase was submaximal at 0·5 μg ml^{-1} of cycloheximide or actinomycin D (Table 1). To ensure maximal increase, the concentration of the drugs used was 5 μg ml^{-1}. When compared with untreated control cells 0·5 and 5 μg ml^{-1} of cycloheximide induced a 1·3 and 2·0-fold increase in alkaline phosphatase activity respectively and actinomycin D induced a 4·4-fold and a 7·8-fold increase. In cells simultaneously incubated with both drugs, enzyme activity was lower than that with actinomycin D alone, but higher than that with cycloheximide alone.

The characteristics of alkaline phosphatases in cells treated with cycloheximide or actinomycin D, were investigated for sensitivity of the enzyme reaction to amino acids such as L-homoarginine and L-phenylalanine (Table 2). Homoarginine was a stronger inhibitor than phenylalanine in three cell samples and in rat liver homogenate. The magnitude of inhibitory effects among these samples was the same. In addition, alkaline phosphatases in three cell samples were heat labile. Both V_{max} and K_m values of alkaline phosphatase for phenylphosphate were increased by cycloheximide or actinomycin D in R-Y121B cells. These results indicate that the drugs alter the characteristics of alkaline phosphatase in R-Y121B cells.

Discussion

The actinomycin D increase in the specific activity of alkaline phosphatase has been termed a 'superinduction', but it is not known whether the enzyme activity is induced (or increased) since actinomycin D decreases the total amount of protein in tissue. A similar induction by actinomycin D was found in tyrosine aminotransferase activity in cultured rat hepatoma cells (Reel & Kenney 1968). 'Superinduction' of enzymes has been explained by speculating on the existence of certain regulators that control enzymes and in our study the 'superinduction' of alkaline phosphatase by actinomycin D was also found in R-Y121B cells. Cycloheximide, which inhibits the translation step in protein synthesis,

Table 2. Characteristics of alkaline phosphatase in the cells, treated or untreated with cycloheximide or actinomycin D, and in the rat liver.

	Homoarginine (% of control) 5 mM	10 mM	Phenylalanine (% of control) 5 mM	10 mM	Heat-inactivation (% of initial) 15 min	30 min	V_{max} (nmol min mg^{-1} protein)	K_m (mM)
Fresh cells	28·7	15·5	86·9	73·5	56·5	36·2	26	1·2
Cycloheximide-treated cells	31·2	18·5	84·7	68·3	68·4	48·0	71	1·8
Actinomycin D-treated cells	33·2	18·2	88·5	73·0	58·4	41·2	125	1·8
Wistar rat liver	36·7	29·1	88·4	67·4	19·3	6·7	5·5	3·8

Cells were treated with cycloheximide or actinomycin D (5 μg ml^{-1}) for 24 h at 37 °C. In the experiments using Wistar rat liver homogenates, where the reaction mixtures were turbid, the absorbance was measured spectrophotometrically after centrifugation of the samples at 10 000 rev min^{-1} for 10 min. The heat inactivation was at 56 °C. The apparent V_{max} and K_m were calculated by Lineweaver-Burk plots.

also increased the total alkaline phosphatase activity. Since this is unusual, we have termed it a 'super-activation' of alkaline phosphatase activity. Although the experiments in-vivo (Moog 1964, 1965; Grey & Moog 1966) showed that puromycin (0·5–10 μg ml^{-1}) also increased the specific activity of alkaline phosphatase, we did not observe a significant increase in its total or specific activity in the presence of puromycin (0·5–10 μg ml^{-1}) in R-Y121B cells (unpublished data).

In our previous study (Sorimachi & Yasumura 1981), cycloheximide at 2·8 μg ml^{-1} completely inhibited the induction of deiodinase, which metabolizes thyroid hormones (Sorimachi & Robbins 1977) and at 5 μg ml^{-1} also completely inhibited the induction of tyrosine aminotransferase activity (unpublished data). In our preliminary observation, 95% of the incorporation of [^{14}C]leucine into cellular proteins was inhibited by cycloheximide at 5 μg ml^{-1}, and the total amount of cellular proteins decreased to ≈60% of the control cells (Table 1). From these results, it seems that protein synthesis at 5 μg ml^{-1} of cyclohexamide could be almost completely inhibited in R-Y121B cells. The 'super-activation' of alkaline phosphatase by cycloheximide does not involve a new protein synthesis, since the drug contained solely in the reaction mixtures did not affect the liberation of phenol from the substrate, nor was the effect of cycloheximide due to a simple allosteric effect of the drug on alkaline phosphatase itself; probably, cycloheximide activates at a very slow rate certain activator(s) which regulate(s) alkaline phosphatase activity.

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