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ALTERATION OF ADENYLATE CYCLASE ACTIVITY BY PHOSPHORYLATION AND DEPHOSPHORYLATION

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Incubation of S49 cell membranes at 0°C resulted in a loss of adenylate cyclase activity, but addition of ATP and ATP regenerating system prevented the decrease of the activity. A non-phosphorylating analogue of ATP, adenyl-5'-yl imidodiphosphate, was less effective than ATP. Treatment of solubilized adenylate cyclase with calf intestine alkaline phosphatase caused the decrease of the activity. Membranes from cyc^- S49 mutant cells, which are devoid of guanine nucleotide-binding protein, yielded the same results as membranes from S49 cells, indicating that the catalytic component is involved in the alteration of the enzyme activity by these treatments. These results suggest that phosphorylation and dephosphorylation of the catalytic component may regulate adenylate cyclase activity.

Protein phosphorylation is known to play an important role in the regulatory process of cellular activity and a number of enzymatic and nonenzymatic proteins are observed to be regulated by phosphorylation and dephosphorylation (1,2). In the case of adenylate cyclase, there have been some reports suggesting regulation by phosphorylation (3-6). Richards et al.(5) proposed that the stimulation of adenylate cyclase by molybdate is related to phosphatase inhibitory properties which fluoride and molybdate share (7-9). They recently reported ATP-dependent activation of adenylate cyclase and suggested that the phosphorylation by ATP may play a role in elevating adenylate cyclase activity (6).

To examine the possibility that phosphorylation and dephosphorylation are involved in the regulation of adenylate cyclase, we studied the effect of incubation of S49 mouse lymphoma cell membranes under conditions where ATP-dependent phosphorylation by an endogenous protein kinase can occur: i.e. incubation with ATP, ATP regenerating system, and Mg^{2+}, and the dephosphorylation by phosphatase treatment. Furthermore, using cyc^- S49 mutant cells (10-13), we sought to identi-

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fy which component of the adenylate cyclase system is involved in the alteration of the enzyme activity by these treatments. The results of these experiments are presented in this report.

MATERIALS AND METHODS

Cell culture: S49 wild type and cyc⁻ mutant cells were grown in Dulbecco's modified Eagle's basal medium supplemented with 10% heat-inactivated horse serum in suspension culture.

Preparation of plasma membrane was according to Courtneidge et al.(14). Adenylate cyclase activity was measured by the method of Salomon et al.(15). Plasma membranes were incubated with ATP as described in the legend to Fig. 1.

Phosphatase treatment of solubilized adenylate cyclase: Adenylate cyclase was solubilized from plasma membranes (2.3 mg protein/ml) with 1% Triton N101 in solution A (20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid]-NaOH pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$). After incubation for 1 h at 0°C, insoluble material was removed by centrifugation at 100,000 × g for 30 min. Extracts were incubated for various times at 0°C with agarose or calf intestine alkaline phosphatase immobilized on agarose (final concentration: 0.76 units/100 µl reaction mixture). Agarose or immobilized alkaline phosphatase was removed by centrifugation and the adenylate cyclase activity of the supernatant was measured.

^{32}P-labeled plasma membranes were prepared by incubation with 20 mM Pipes [1,4-piperazinediethanesulphonic acid]-NaOH pH 7.2, 4 mM $MgCl_2$, and [γ-^{32}P]ATP (1 µM, 63 mCi/µmole) for 20 min at 30°C. ^{32}P-labeled plasma membranes collected by centrifugation at 100,000 × g for 30 min were solubilized and treated with agarose or immobilized alkaline phosphatase as described above. Radioactivity not removed from proteins was measured according to the procedure of Cassel et al.(16). Immobilized alkaline phosphatase was obtained from Sigma.

RESULTS

Plasma membranes prepared from S49 mouse lymphoma cells were incubated with or without ATP, the ATP regenerating system and $MgCl_2$ at 0°C for various times. As shown in Fig. 1(a), incubation of plasma membranes without ATP and ATP regenerating system resulted in a loss of adenylate cyclase activity in a time-dependent manner. By contrast, plasma membranes incubated with ATP and ATP regenerating system did not exhibit this loss. When plasma membranes were incubated with a non-phosphorylating analogue of ATP, AppNHp [adenyl-5'-yl imidodiphosphate], adenylate cyclase activity decreased (Table 1).

To examine the possibility that adenylate cyclase activity is changed by dephosphorylation, adenylate cyclase solubilized with Triton N101 was reacted with immobilized calf intestine alkaline phosphatase, and after incubation the solubilized adenylate cyclase and immobilized alkaline phosphatase were separated by centrifugation. As shown in Fig. 2(a), adenylate cyclase activity was decreased by the treatment with alkaline phosphatase. By contrast, adenylate cyclase incu-

bated with agarose alone showed no decrease of activity. To verify the removal of phosphate from solubilized membrane proteins by this reaction, the plasma membrane phosphorylated with [γ-^{32}P]ATP was solubilized and treated with immobilized alkaline phosphatase and ^{32}P radioactivity was measured. As shown in Fig. 3(a), ^{32}P radioactivity of proteins was removed by this treatment in a time-dependent manner.

To identify which component of adenylate cyclase system is involved in the alteration of the activity by the treatment described above, we prepared plasma membrane from cyc$^-$ variant cells which lack guanine nucleotide-binding protein and examined the alteration of adenylate cyclase activity by the incubation with ATP and the treatment with alkaline phosphatase. Adenylate cyclase activity was assayed in the presence of Mn^{2+}. As shown in Fig. 1(b), the response to incubation with ATP was similar to S49 adenylate cyclase. Phosphatase treatment resulted in the decrease of the activity and concurrently phosphate was removed from the reacted proteins (Fig. 2(b), 3(b)).

DISCUSSION

In this report we have studied the possibility that phosphorylation and dephosphorylation are involved in the regulation of adenylate cyclase activity. As the purification of the adenylate cyclase system is difficult to achieve for the present, studies with purified molecules and detailed analysis at the molecular level are impossible. Therefore we studied this enzyme system by investigating the alteration of the activity by incubation of plasma membrane with ATP and phosphatase.

While incubation of plasma membrane of S49 cells without ATP and ATP regenerating system resulted in a loss of adenylate cyclase activity, incubation with ATP, ATP regenerating system, and Mg^{2+} prevented such a loss of activity (Fig. 1(a)). A non-phosphorylating analogue of ATP, AppNHp, was not as effective as ATP (Table 1). Though Richards et al. reported that incubation of rat liver plasma membrane with ATP, ATP regenerating system, and Mg^{2+} results in a 4-7 fold activation of adenylate cyclase, we did not observe this stimulation. Membrane fractions prepared from other cell lines (rat normal 3Y1 cells and adenovirus type 12-transformed 3Y1 cells) by various methods yielded similar results (data not shown).

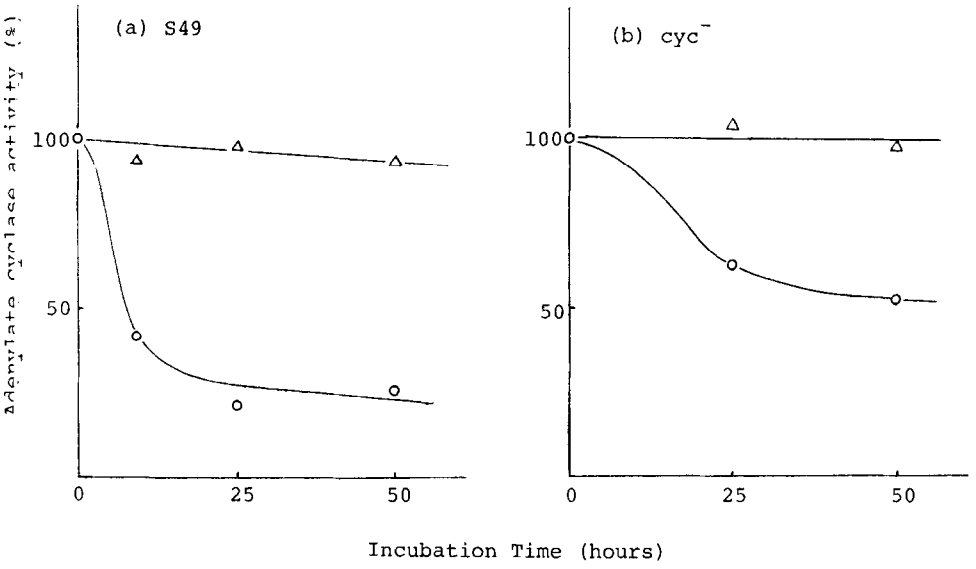

Fig. 1. Effect of incubation with ATP and ATP regenerating system on adenylate cyclase activity. Plasma membranes of S49 cells (a), or cyc⁻ cells (b) (1.5 mg protein/ml, final concentration) were incubated with 50 mM Hepes-NaOH pH 7.4, 1 mM ATP, 10 mg/ml phosphocreatine, 5 mg/ml phosphocreatine kinase, and 5 mM $MgCl_2$ (o), or with 50 mM Hepes-NaOH pH 7.4 and 5 mM $MgCl_2$ (Δ) at 0°C and then diluted with 6 volumes of 20 mM Hepes-NaOH pH 7.4, and centrifuged at 100,000 × g for 30 min. The obtained membrane pellets were used for the assay of adenylate cyclase activity. 100% is 10.5 pmol/mg protein/min in (a) and 5.1 pmol/mg protein/min in (b).

One possible interpretation of these results is as follows: Removal of phosphate from adenylate cyclase by an endogenous phosphatase activity during the incubation without ATP and ATP regenerating system resulted in the decrease in adenylate

Table 1

Incubation of S49 cell plasma membranes with ATP or AppNHp

Condition of incubation	adenylate cyclase activity pmol/mg protein/min	(% of initial activity)
ATP (1 mM), ATP regenerating system	9.5	(96)
AppNHp (1 mM), ATP regenerating system	3.9	(39)
—	3.0	(30)

S49 cell plasma membranes (1.5 mg protein/ml, final concentration) were incubated with 50 mM Hepes-NaOH pH 7.4, 5 mM $MgCl_2$, and other components for 20 h at 0°C. Membranes were recovered from incubation and assayed for adenylate cyclase activity as described in Materials and Methods. ATP regenerating system consists of phosphocreatine and phosphocreatine kinase (final 10 mg/ml and 5 mg/ml, respectively). The initial adenylate cyclase activity of the membrane preparation was 9.9 pmol/mg protein/min.

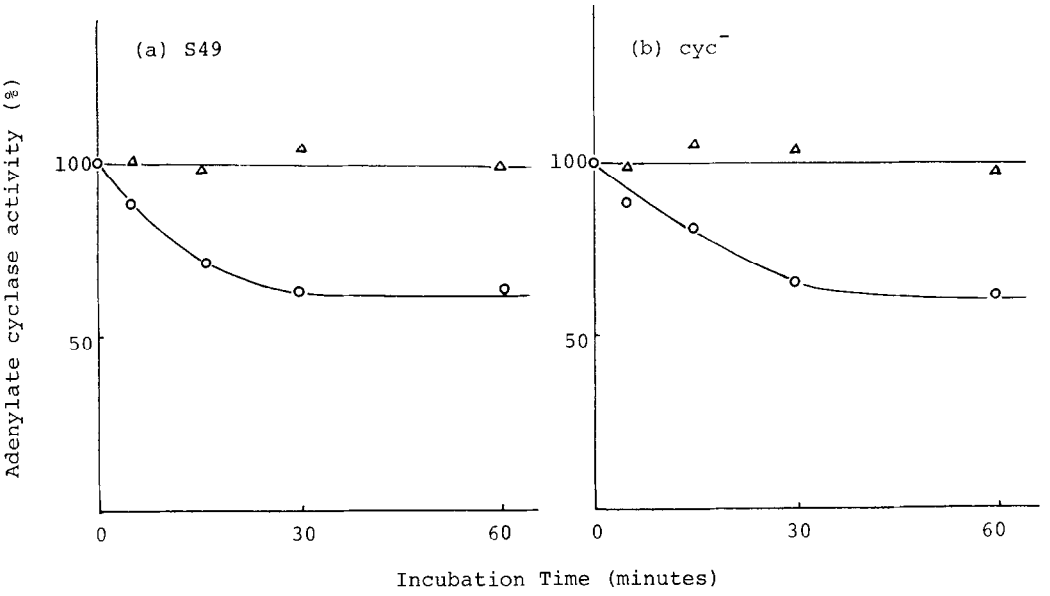

Fig. 2. Effect of alkaline phosphatase treatment on adenylate cyclase activity. Adenylate cyclase was solubilized from plasma membranes of S49 cells (a), or cyc⁻ cells (b) (2.3 mg protein/ml) with 1% Triton N101 as described in Materials and Methods. The solubilized adenylate cyclase (80 μg protein) was reacted with 0.76 units of immobilized calf intestine alkaline phosphatase (o) or with agarose (Δ) at 0°C in a total volume of 100 μl. After centrifugation the obtained supernatant was assayed for adenylate cyclase activity in the presence of 50 μM guanyl-5'-yl imidodiphosphate (a) or 5 mM $MnCl_2$ (b). 100% is 12.4 pmol/mg protein/min in (a) and 7.3 pmol/mg protein/min in (b).

cyclase activity. When ATP and ATP regenerating system were added, phosphorylation by an endogenous protein kinase competed with the endogenous phosphatase activity and prevented a loss of adenylate cyclase activity.

This model was supported by the effect of dephosphorylation by added immobilized phosphatase. In order to exclude an effect of phosphatase on other components of the memebrane: e.g., phospholipids, we used adenylate cyclase solubilized from plasma membrane. In this system, decrease of the adenylate cyclase activity was observed in parallel with the dephosphorylation of the proteins (Fig. 2(a), 3(a)). These results suggest that the activity of adenylate cyclase can be changed by removal of phosphate.

It is known that adenylate cyclase systems consist of at least three separable components: a hormone receptor, a guanine nucleotide-binding regulatory component, and a catalytic component (17, 18). We employed cyc⁻ mutant S49 cells to identify the component involved in the alteration of adenylate cyclase activity by the

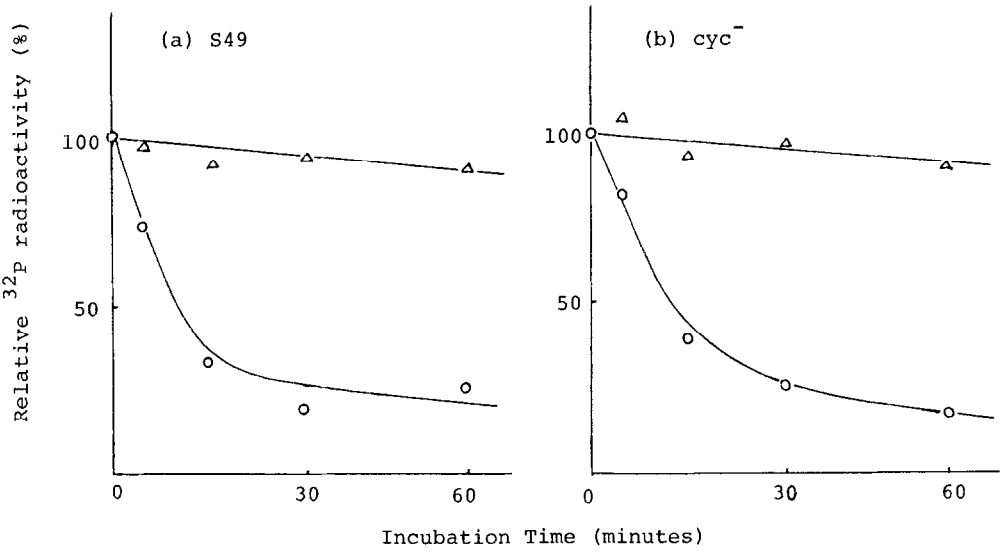

Fig. 3. Time course of dephosphorylation of ^{32}P-labeled solubilized membrane proteins. Plasma membranes (2.3 mg protein/ml) were phosphorylated with [γ-^{32}P]ATP and solubilized with 1% Triton N101 as described in Materials and Methods. The solubilized adenylate cyclase (80 µg protein) was incubated with 0.76 units of immobilized calf intestine alkaline phosphatase (○) or with agarose (△) at 0°C in a total volume of 100 µl. At the indicated times samples were centrifuged and the supernatant was used for the determination of ^{32}P radioactivity associated with proteins. (a) S49 (100% = 2101 cpm), (b) cyc⁻ (100% = 1902 cpm).

treatment described above. The cyc⁻ mutant of S49 mouse lymphoma cells has a defective guanine nucleotide-binding regulatory component and is unable to respond to stimulation of hormones, guanine nucleotides or fluoride ion and the activity of the catalytic component alone can be measured in the presence of Mn^{2+} (10-13). Using these mutant cells, the catalytic component was shown to exhibit the same response as adenylate cyclase of wild type S49 cells (Fig. 1(b), 2(b), 3(b)). As the activity of the solubilized catalytic component of cyc⁻ cells has no relevance to hormone receptors and guanine nucleotide-binding component, the catalytic component was assumed to be the site of action of the treatment. The results of these experiments indicate that the activity of the catalytic component may be regulated by phosphorylation and dephosphorylation.

The identity of the protein kinase and whether phosphorylation by a purified protein kinase can enhance the decreased activity of dephosphorylated adenylate cyclase remain to be determined. Final proof of the hypothesis will require purification and characterization of the catalytic component and the protein kinase

involved. Analysis of protein kinases of the plasma membrane revealed the presence of several memebrane-associated protein kinases and each of which phosphorylated specific membrane proteins (unpublished data). In relation to the phosphorylation of adenylate cyclase, characterization of these protein kinases is under investigation.

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