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Mechanism of Glycogenolytic Action of Cycloheximide in Rat Liver*

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SUMMARY

Cycloheximide (0.1 to 0.2 mM) increases cAMP concentration 3 to 4-fold in isolated rat liver slices in vitro. This increase in cAMP concentration parallels an increase in phosphorylase activity. When cycloheximide, at a concentration used to inhibit protein synthesis (1 to 2 μg/g body weight), is administered to whole animals, phosphorylase is activated up to 13-fold by 6 hours. This leads to almost complete depletion of liver glycogen (from about 40 mg/g liver to 0.4 mg/g liver).

INTRODUCTION

Cycloheximide, a potent inhibitor of protein synthesis has been used widely to ascertain whether protein synthesis is involved in the action of hormones on the assumption that the sole effect of cycloheximide is to inhibit protein synthesis. However, use of cycloheximide, particularly in whole animals, often produces some anomalous effects which cannot be attributed solely to inhibition of protein synthesis. One of these anomalous effects which is independent of protein synthesis is cycloheximide's very potent glycogenolytic action in the liver. Such stimulated glycogenolysis results in changes in the concentration of cellular metabolites and thus affect the activity of many enzymes (1).

To elucidate the mechanism by which cycloheximide activates glycogenolysis, we have examined the effect of the drug in isolated rat liver slices as well as in whole animals.

In this communication, we report results which indicate that cycloheximide itself increases cAMP concentration 3 to 4-fold. This stimulation is accompanied by the same degree of phosphorylase activation.

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MATERIALS AND METHODS

Chemicals - Shellfish glycogen, glucose-1-P, cycloheximide and 5'-AMP were the products of Sigma Chemical Co. Theophylline and cAMP assay kits were purchased from Calbiochem. [^{14}C]glucose-1-P (sp. act. 200 mC/mmol) and [^{3}H]cAMP (sp. act. 81 C/mmol) were obtained from Schwarz/Mann, Inc. Glucagon was the product of Eli Lilly Pharmaceuticals.

Animals - Male Wistar rats weighting 200-250 g were from the Department of Biochemistry animal colony.

Tissue Preparation and Enzyme Assays - Liver slices were prepared using a Stadie-Riggs microtome. The livers were removed immediately after decapitation and kept in cold Krebs-Ringer solution. The tissues were cut into circles of 1.5 cm diameter with a sharp cork-screw borer and then cut into slices of 0.5 mm thickness with the tissue slicer. All liver slices used were of the same diameter and thickness. Each culture flask, containing 4 slices of tissue (300 mg) in 5 ml Krebs-Ringer medium pH 7.4, was treated as described by Taylor et al. (2). The slices were maintained under atmosphere of 95% O_2 and 5% CO_2 during both the preincubation and incubation periods. Glucagon and cycloheximide treatments were carried out in the presence of 1 mM theophylline. At the end of the incubation period the slices were removed and washed with cold Krebs-Ringer solution. The tissues were homogenized in cold buffer (3 ml per g tissue) containing 0.25 M sucrose, 50 mM glycerophosphate, pH 6.8, and 1 mM EDTA. The supernatant fraction was obtained for glycogen phosphorylase assay by centrifugation at 12,000 x g for 10 min at 4°C.

Glycogen Phosphorylase Assay - Glycogen phosphorylase was assayed by determining the incorporation of radioactive glucose into glycogen from [^{14}C] glucose-1-P. The standard assay mixture contained 2 mg glycogen, 2 µmol [^{14}C] glucose-1-P (16,000 cpm), 50 µmol β-glycerophosphate, pH 6.8, 0.5 µmol EDTA, and 0.1 ml of supernatant, in a final volume of 0.5 ml. The reaction mixture was incubated at 30° for 10 minutes in the absence of 5'-AMP.

Tissue Preparation and cAMP Assay - Treated liver slices were dipped into liquid nitrogen at the end of incubation period. The tissues were then homogenized at 0°C in 2 ml of 0.5 M HCl containing 66% ethanol. The acid solutions were then centrifuged at 12,000 x g for 10 min at 4°C. Under these conditions, glycogen was completely removed from the supernatant. Aliquots of the acid soluble material were then dessicated to dryness. The dried samples were dissolved in a small volume of 0.05 M sodium acetate buffer, pH 4.0, and assayed for cAMP as described (3).

RESULTS AND DISCUSSION

The time course of the cycloheximide effect on liver glycogen levels in the whole animal is shown in Fig. 1. The drug exerted its maximal effect after six hours, at which time the glycogen content dropped from 37.3 mg/g liver in the control to 0.4 mg/g liver. The response was rapid, a substantial effect being seen at two hours (a drop of almost 70%). After eight hours, the glycogen content started increasing, indicating that the effect of the drug was beginning to disappear.

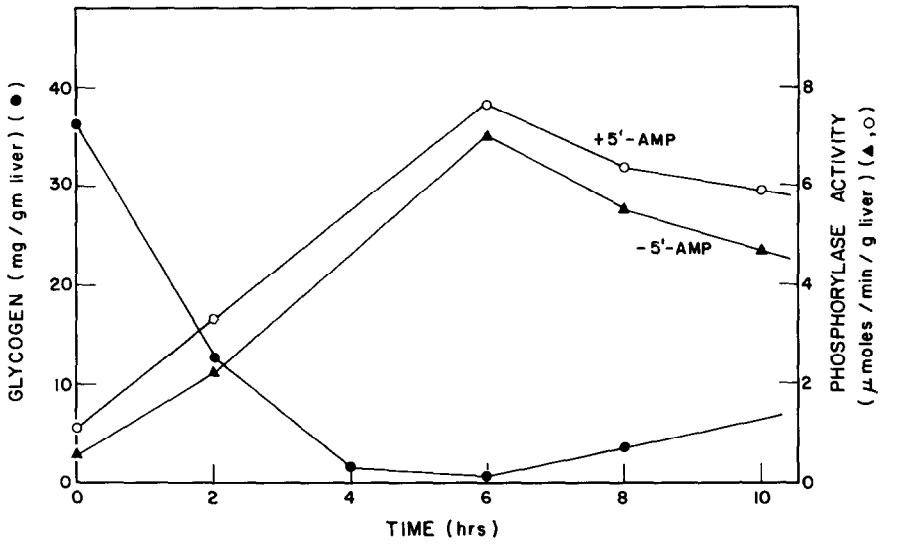
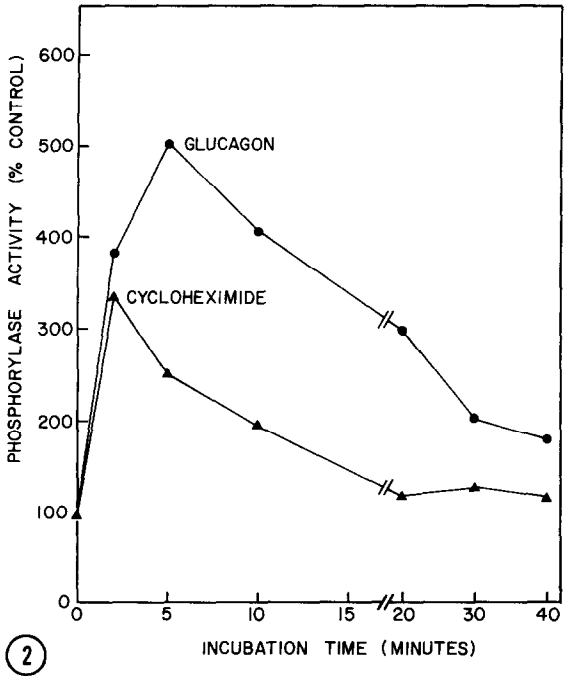


Fig. 1. Effect of Cycloheximide on Glycogen Level and Glycogen Phosphorylase. Rats were injected intraperitoneally with 1.25 μg/gm body weight of cycloheximide, killed at the times indicated, and glycogen prepared from the livers was determined by the phenol/sulfuric acid method. For the glycogen phosphorylase assay, animals treated with cycloheximide as described above were used. Glycogen phosphorylase was assayed in the presence (○) and absence (▲) of 2mM 5'-AMP. Each experimental point represents the value from three pooled livers.

Since phosphorylase is a key enzyme in the mobilization of glycogen, the effect of cycloheximide on phosphorylase activity was examined (Fig. 1). The drug showed a maximal effect at six hours with an increase in phosphorylase activity of more than 13-fold in both the presence and absence of 5'-AMP. At longer time periods, phosphorylase activity decreased. This result corresponded well with the data on glycogen content which showed minimal glycogen levels when phosphorylase was maximally activated. The rapid response of enzyme to the drug was indicated by a four-fold increase in enzyme activity at two hours.

Liver phosphorylase b is only slightly activated by 5'-AMP (4) compared to muscle phosphorylase b, which requires 5'-AMP for the activity. The possibility that the increase in enzyme activity was due to the accumulation of 5'-AMP in the liver cells could be eliminated. Comparison of phosphorylase activities in the homogenates before and after charcoal treatment to remove 5'-AMP showed no change in activity. Also, the activated phosphorylase retained its activity following passage through a column of Sephadex G-25 indicating that no small cellular metabolites are involved in the activiation.

To eliminate any ambiguities arising from the use of the whole animal, we have examined the effect of cycloheximide on isolated liver slices *in vitro*. In Fig. 2, the effect of 0.1 mM cycloheximide and 0.1 μM glucagon on the activation of phosphorylase is shown. Glucagon



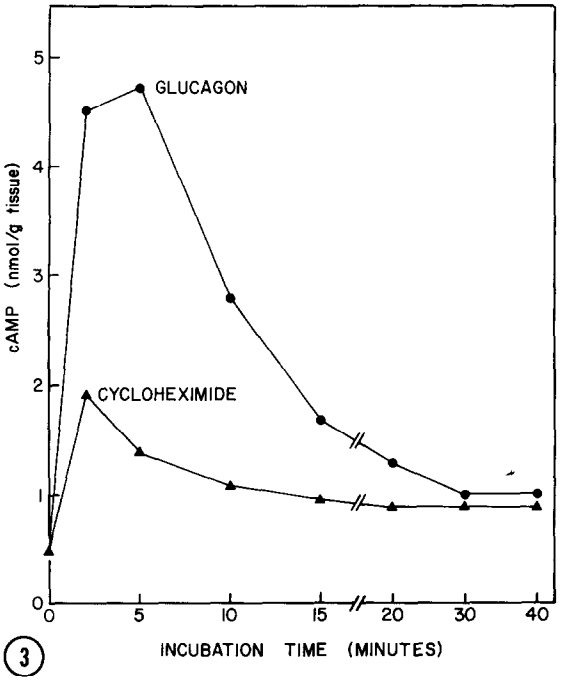


Fig. 2. Effect of Glucagon and Cycloheximide Incubation in vitro on Phosphorylase a Activity of Liver Slices. Rat liver slices were prepared and treated in the same manner as described in Materials and Methods. Tissue slices treated with either 0.1 μM glucagon or 0.1 mM cycloheximide were homogenized in 3 volumes of 0.25 M sucrose, 50 mM glycerolphosphate, pH 6.8, and 1 mM EDTA. The homogenates were centrifuged at 12,000 x g for 10 min and the supernatants used for the assay of phosphorylase a activity as described in the Methods.

Fig. 3. Effect of Glucagon and Cycloheximide on cAMP Level of Liver Slices in vitro. Tissue slices were prepared as described in Methods. Four slices (~300 mg tissue) were used in each incubation flask containing 5 ml Krebs-Ringer solution. The slices were preincubated at 37°C for 10 min under an atmosphere of 95% O_2, 5% CO_2 before incubation with 0.1 μM glucagon or 0.1 mM cycloheximide was begun. At the end of each incubation period the tissues were removed, washed with cold Krebs-Ringer solution and snap-frozen in liquid nitrogen. The treated tissues were then homogenized at 0°C in 2 ml of 0.5 M HCl containing 66% ethanol. The homogenates were centrifuged at 12,000 x g for 10 min and supernatants collected. Aliquots of the acid soluble material were dessicated to dryness. The dried samples were dissolved in the same volume of 0.05 M sodium acetate buffer, pH 4.0, and assaye for cAMP content as described (3).

activates phosphorylase over 5-fold by 5 min while cycloheximide activates over 3-fold at 2 min. Similar kinetics of activation were observed in several experiments and the peak of the cycloheximide effect was observed at 2 min while that of glucagon occurred at 5 min.

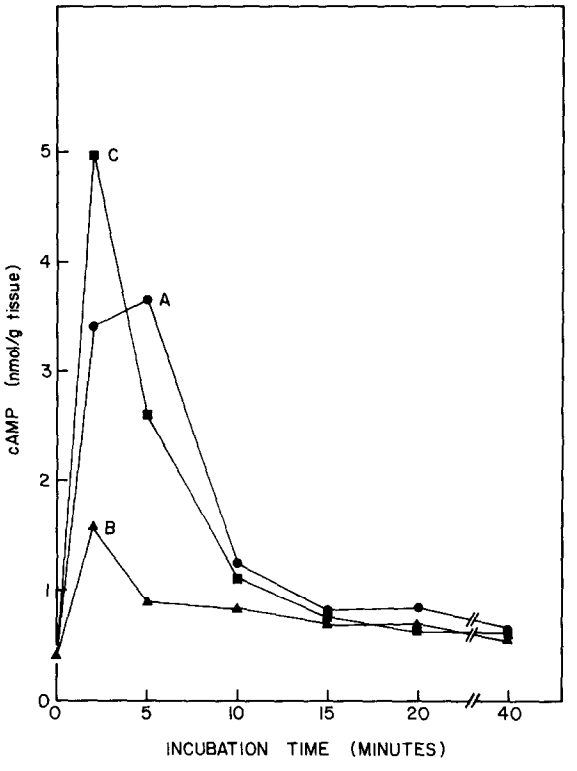


Fig. 4. Combined Effect of Glucagon and Clycoheximide on cAMP Level of Liver Slices in vitro. The conditions were as described under Fig. 3. Glucagon and Cycloheximide were added simultaneously in this study.

A 0.1 μM glucagon
B 0.1 mM cycloheximide
C 0.1 μM glucagon + 0.1 mM cycloheximide

The effect of these two agents on the level of cAMP is shown in Fig. 3. In parallel to those effects on the phosphorylase activity, cycloheximide and glucagon increased the level of cAMP. Glucagon caused an increase of about 8-fold in a 2 to 5 min period whereas cycloheximide increased the cAMP level about 3-fold at 2 min. These increases in the cAMP concentration can be observed only in the presence of theophylline.

We have previously reported that increases in the cAMP level caused by cycloheximide in the isolated epididymal fat pad were due to cycloheximide acting at the same site as epinephrine (5). However, simultaneous addition of cycloheximide and glucagon caused a synergistic effect on the level of cAMP in the liver slices as shown in Fig. 4.

The data presented in this brief note indicate that the glycogenolytic effect of cycloheximide is the result of an increased cAMP

level in the tissue which leads to the subsequent activation of phosphorylase. Therefore, any experiments utilizing cycloheximide should be cautiously evaluated in view of the drug's extremely rapid effect on cyclic nucleotide metabolism and glycogenolysis. These effects may profoundly disturb the balance of cellular metabolites.

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