EFFECT OF CYCLOHEXIMIDE ON ADENOSINE 3':5'-MONOPHOSPHATE LEVEL IN RAT EPIDIDYMAL FAT TISSUE*

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Summary: Cycloheximide at 0.1 to 0.2 mM increases cAMP concentration up to five-fold in epididymal fat tissue in vitro. This increase in cAMP concentration is accompanied by a 40% activation of glycogen phosphorylase. Propranolol, a specific β-adrenergic antagonist, blocks the cycloheximide-mediated cAMP increase. Epinephrine stimulates cAMP formation up to 25-fold under the same condition. This increase is also blocked by propranolol. Cycloheximide also partially blocks the epinephrine stimulated cAMP increase, suggesting that both compounds act at the same site.

Cycloheximide, a potent reversible inhibitor of protein synthesis, is known to inhibit the peptidyl transferase activity of the 60s ribosomal subunit of eucaryotes (1). Thus cycloheximide has been widely used 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. Nevertheless, some anomalous effects of cycloheximide have been observed when the drug is used in whole animals where it is difficult to distinguish between primary and secondary effects of cycloheximide (2-4).

Recently, we have observed that cycloheximide is a very potent glycogenolytic agent and that this effect is independent of protein synthesis (4). For example, cycloheximide treatment (1.25 μg/g body weight) was accompanied by a large activation of liver glycogen phosphorylase and concomitant depletion

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of liver glycogen (from about 40 mg/g liver to 0.4 mg). This action of cycloheximide was reversible.

In an attempt to elucidate the mechanism by which cycloheximide activates glycogen phosphorylase, we have examined the effect of the drug in isolated epididymal fat tissue. The complications arising from using the whole animal are largely eliminated with this in vitro system.

In this communication, we report experiments which show that cycloheximide itself affects cAMP metabolism through the adrenergic β-receptor site.

MATERIALS AND METHODS

Chemicals - Shell fish glycogen, glucose-1-P, cycloheximide and epinephrine 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/m mol) and [^{3}H]-cAMP (sp. act. 81 c/m mol) were obtained from Schwarz/Mann, Inc. Propranolol was the product of Ayerst Lab., New York.

Animals - Male Wistar rats weighing 175-225 g were from the Department of Biochemistry's animal colony.

Tissue Preparation and Enzyme Assays - To avoid individual variations, the epididymal fat tissues from at least 20 animals were combined. The tissue was cut into pieces approximately 2 mm^3. Each culture flask contained 300 mg of tissue and was treated as described by Taylor et al., (5) except that epinephrine and cycloheximide treatments were carried out in the presence of 3% dialyzed bovine serum albumin. All incubation mixtures contained 1 mM theophylline. After incubation, the tissues were washed with Krebs-Ringer solution and homogenized in cold buffer (5 ml per g tissue) with a glass homogenizer. The homogenization buffer contained 0.25 M sucrose, 50 mM glycerophosphate, pH 6.8 and 1 mM EDTA. The infranatant fraction was obtained for glycogen phosphorylase assay by centrifugation at 12,000 x g for 10 min at 4°.

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.

To assay cAMP levels, treated tissues were added directly to 1.2 ml of 0.1 N HCl to terminate the reaction. The tissues were then digested in the acid for 25 min in a boiling water bath. Under these conditions, the tissue was completely disintegrated. The acid solutions were then centrifuged at 4000 rpm for 10 min at 4°. Aliquots of the acid soluble material were lyophilized. The dried samples were dissolved in a small volume of 0.05 M sodium acetate buffer, pH 4.0, and assayed as described (6). Each sample was determined in triplicate. All values reported are the average of these determinations.

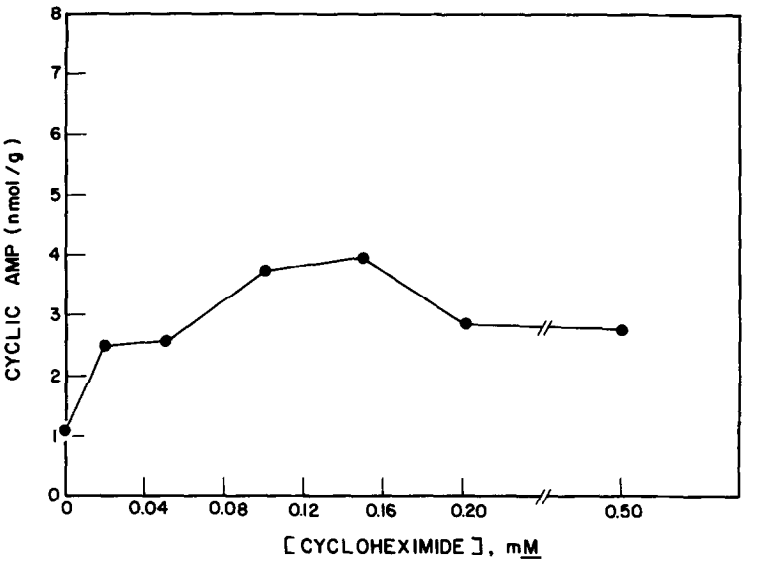

Fig. 1. Effect of cycloheximide concentration on cAMP accumulation of epididymal fat tissue. The fat pads were removed from young male rats immediately after decapitation. They were pooled and cut into small pieces. 300 mg of the pads were preincubated in 5 ml of Krebs-Ringer bicarbonate media, pH 7.4, containing 3% BSA and 1 mM theophylline, at 37° for 20 minutes under 95% O_2, 5% CO_2. At the end of this period, different concentrations of cycloheximide were added to the incubation flasks and the incubation was continued for 10 minutes. The reaction was terminated by transferring the tissue from media into 1.2 ml of 0.1 N HCl and immediately mixing on Vertex mixer. The acidified tissues were heated in the boiling water bath for 25 minutes. Under this condition the fat pads were completely digested. The fat cake was separated from acid soluble supernatant by centrifugation at 4000 RPM for 10 min at 4°. 0.3 ml of the supernatant was lyophilized to remove HCl and the dried samples were dissolved in 0.6 ml of 0.05 M sodium acetate buffer, pH 4.0. 50 µl of each reconstituted sample were assayed in triplicate for the cAMP content.

RESULTS AND DISCUSSION

The effect of cycloheximide concentrations on cAMP level in isolated epididymal fat tissue is shown in Fig. 1. In this experiment, tissue was treated with cycloheximide for 10 min in the presence of 1 mM theophylline. In the absence of theophylline, little or no increase in cAMP concentration was observed. Theophylline exerted the same effect when tissue samples were incubated with epinephrine. Although theophylline itself had no effect on the concentration of cAMP or glycogen physphorylase activity, its presence was required for manifestation of the effect of cycloheximide or epinephrine. In subsequent experiments, 1 mM theophylline was always included in the incubation mixture.

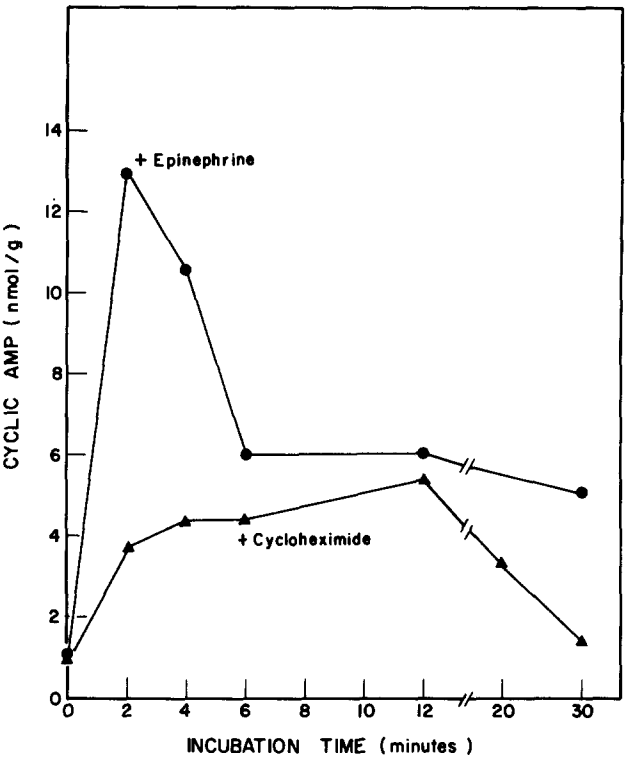

Fig. 2. The time course of the effect of epinephine and cycloheximide on cAMP accumulation of fat pad. The conditions were as described under Fig. 1. Epinephrine (4×10^{-5} M) and cycloheximide (2×10^{-4} M) were added to the incubation flasks at appropriate times after 20 minutes preincubation at 37° under 95% O_2 and 5% CO_2.

The maximum level of cAMP was observed with 0.15 mM cycloheximide. Higher concentrations of the drug were not as effective.

The time course of cAMP formation in the presence of either 4×10^{-5} M epinephrine or 0.20 mM cycloheximide is shown in Fig. 2. Epinephrine immediately stimulated cAMP formation with a 13-fold elevation observed after 2 min, but the increased level of cAMP decayed rapidly over a longer period. Similar results have been reported by Ho and Sutherland who explained this decrease on the basis of the appearance of a hormone antagonist (7). In contrast, the five-fold increased level of cAMP due to cycloheximide treatment was maintained for 10 to 12 min.

It is reasonable to assume that the prolonged existence of a large cAMP pool may occur because in the presence of cycloheximide the drug is unable

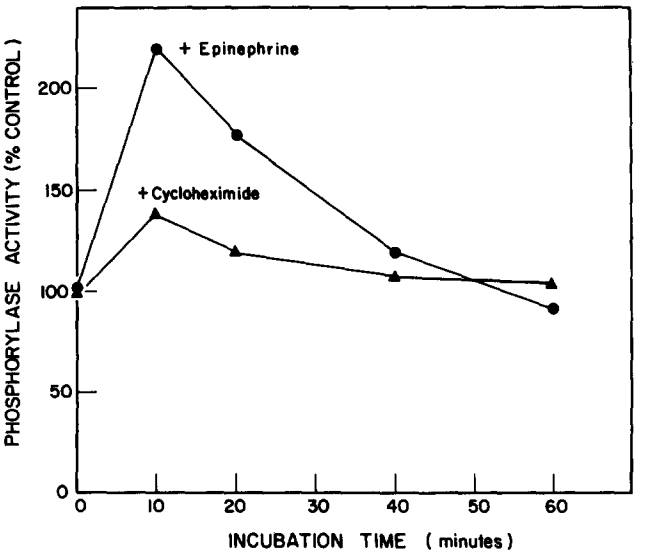

Fig. 3. The time course of this effect of epinephine and cycloheximide on glycogen phosphorylase activity of fat pads. The conditions were as described under Fig. 1. Epinephrine (4×10^{-5} M) and cycloheximide (1.5×10^{-4} M) were added to the incubation flasks at 37° under 95% O_2 and 5% CO_2. At the end of each incubation period, the tissues were removed, washed in fresh Krebs-Ringer bicarbonate media and homogenized in 5 volume of glycerophosphate buffer, pH 6.8. The enzyme activity was expressed as percentage of activity in the control tissue.

to induce the formation of sufficient hormone antagonist that is elicited so quickly by epinephrine (7). Recent report indicates that the antagonists are long chain fatty acids (8).

Both epinephrine and cycloheximide activated glycogen phosphorylase, as shown in Fig. 3. Epinephrine activated glycogen phosphorylase about 2-fold, whereas cycloheximide activated the enzyme to a smaller extent (about 40%). These experiments indicate that both agents increase the cAMP pool and thus activate glycogen phosphorylase.

Since the activation of phosphorylase by cAMP is mediated through the adrenergic β-receptor, the effect of propranolol, a specific β-receptor blocking agent, was examined. As shown in Fig. 4, propranolol completely inhibited the increase in the cAMP concentration by either cycloheximide or epinephrine. In this experiment, cycloheximide alone had increased the cAMP

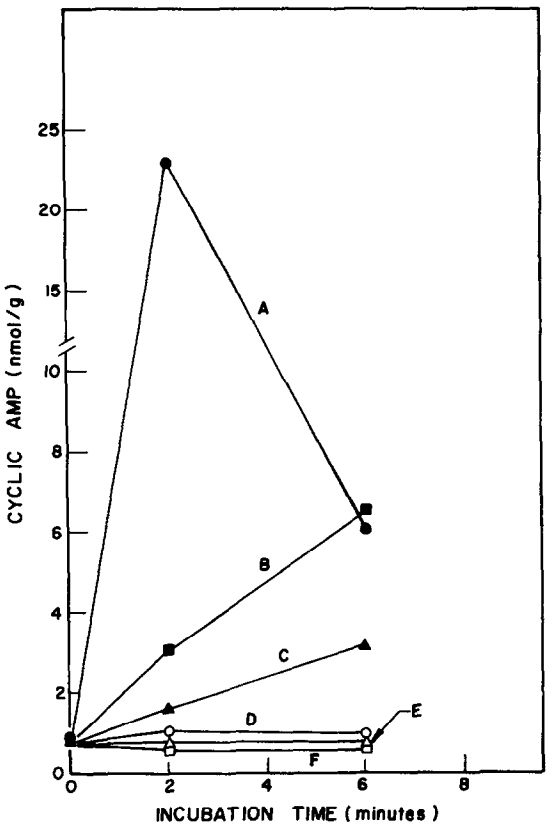

Fig. 4. The effect of propranolol and cycloheximide on cAMP accumulation in fat pad stimulated by epinerphrine. The conditions were as described under Fig. 1. Propranolol and cycloheximide were added simultaneously with epinephrine in these studies.

A. 4×10^{-5} M epinephrine
B. 4×10^{-4} M epinephrine + 1.5×10^{-4} M cycloheximide
C. 1.5×10^{-5} M cycloheximide
D. 4×10^{-4} M epinephrine + 1×10^{-5} M propranolol
E. 1.5×10^{-5} M cycloheximide + 1×10^{-5} M propranolol
F. 1×10^{-5} M propranolol

concentration about 2-fold after 2 min and more than 3-fold after 6 min, while epinephrine alone had increased the cAMP level about 25 fold after 2 min.

It is interesting to note that the addition of epinephrine together with cycloheximide resulted in a level of cAMP at 2 min significantly lower than that observed when epinephrine acted alone. After 6 min, the cAMP concentration was about the same as that observed with epinephrine alone. This suggests that the apparent inhibition of epinephrine-stimulated cAMP formation

may result from competition between cycloheximide and the hormone for the same site. This inference is also supported by the observation that propranolol blocks the stimulations of cAMP formation by either epinephrine or cycloheximide.

In a recent report, we showed that cycloheximide (1.25 μg/g body weight) activated hepatic phosphorylase activity up to 13-fold after 6 hours (4). If this enzyme activation is solely due to the effect of the drug on the formation of cAMP, the magnitude of the cAMP increase in the liver must be extremely high. For technical reasons, we have used epididymal fat tissue instead of hepatic tissue for the *in vitro* studies. The results obtained with the isolated tissue clearly indicate that cycloheximide directly affects cAMP metabolism.

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