# EFFECTS OF HORMONES ON CYCLIC AMP RELEASE FROM RAT ADIPOSE TISSUE IN VITRO

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## 1. Introduction

Lipolysis is regulated, at least in part, by the intracellular level of cyclic AMP [1,2]. The effects of several hormones and of lipolytic and antilipolytic agents on cAMP accumulation in adipose tissue and adipocytes have been extensively studied [3–6]. Most experiments were carried out with isolated fat cells [4–8]. Little attention has been paid to cAMP release from intact adipose tissue [3].

During liver perfusion cAMP is released into the perfusion medium in response to glucagon and to epinephrine [9,10]. Therefore, we examined to what extent cAMP was released from adipose tissue in response to these lipolytic hormones and whether lipolysis was correlated with cAMP release, which may reflect metabolically available 'free' cAMP in the cell [9]. Furthermore, we were interested to find out whether partially purified nonsuppressible insulin-like activity (NSILA-S) which has been shown to exert the same metabolic effects on adipose tissue as insulin (for review see [11]) affects epinephrine-induced cAMP release. The results of this study show that: 1) Considerable amounts of cAMP are released from adipose tissue only when theophylline is present in addition to lipolytic hormones. After 30 min cAMP, which has accumulated in the medium, rapidly disappears possibly due to enhanced degradation. 2) cAMP release is inhibited by insulin and NSILA-S. 3) Concomitant inhibition by insulin of epinephrine-induced lipolysis and of cAMP release is observed only at low epinephrine concentrations, whereas at higher epinephrine concentrations lipolysis is no longer inhibited by insulin, although cAMP-release is reduced.

## 2. Materials and methods

Male Osborne-Mendel rats weighed 140–160 g and were fed with normal chow. After decapitation the epididymal fat pads were rapidly excised and pooled (200–500 mg per flasks). The flasks contained 2.5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, with 200 mg of human serum albumin per 100 ml. After 10 min of preincubation, hormones or other agents were added. At the end of the incubation adipose tissue was rapidly separated from the medium by pouring it over nylon stocking. cAMP was determined in the medium by the competitive protein binding assay of Gilman [12] modified by Brown [13] as previously described [10]. Each assay tube contained: 0.2 ml of 100 mM Tris–HCl buffer, pH 7.5, with 16 mM theophylline and 20 mM magnesium chloride, 0.05 ml of [$^3$H]cAMP (50 nCi, specific activity 20.7 Ci/nmole, the Radiochemical Center, Amersham), 0.05 ml of binding protein solution and 0.2 ml of incubation medium. 0.2 ml of Krebs-Ringer bicarbonate buffer containing 200 mg of human serum albumin per 100 ml and between 0–10 pmoles of cold cAMP (Boehringer, Mannheim) were used for standards.

After incubation for 60 min at 0°C, 0.5 ml of a charcoal–albumin suspension (5 g of Norit A, Serva, 0.9 g of human serum albumin and 100 ml of 50 mM Tris–HCl buffer, pH 7.5, containing 8 mM theophylline and 10 mM magnesium chloride, stirred for at least 2 hr at 0°C) was added to each assay tube. Incubation was continued for 30 min at 0°C and the tubes were

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then centrifuged for 15 min at 0°C and 3000 *g*. 0.5 ml of the supernatant was added to 5 ml of Instagel (Packard) and counted in a liquid scintillation counter (Unilux, Nuclear, Chicago).

The cAMP binding protein was prepared from rat livers. After injecting Tris–EDTA buffer, pH 7.5 (50 mM Tris–HCl, 4 mM EDTA), into the hepatic veins to wash out the blood, livers were homogenized in two vols of the same buffer and the homogenate was centrifuged for 15 min at 30 000 *g*. 1 ml aliquots of the supernatant were lyophilized and used after a 1:4 dilution with distilled water.

Glycerol was determined by the method of Eggstein and Kreutz [14]. Samples were deproteinized with perchloric acid (3% final concentration) and neutralized with solid $KHCO_3$ prior to determination. NSILA-S, 15 mU/mg as standarized by the stimulation of net gas exchange in the fat pad assay [15] was provided by R. E. Humbel [16].

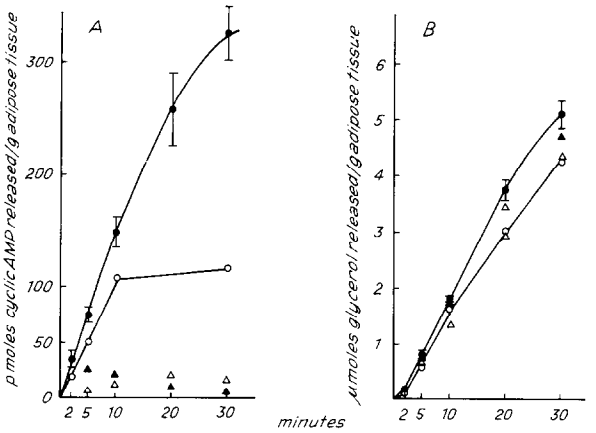


Fig. 1. Effect of epinephrine and glucagon on cAMP (A) and glycerol release (B) into the incubation medium. After preincubation for 10 min either epinephrine (0.5 μg/ml) (▲–▲–▲) or glucagon (5 μg/ml) (△–△–△) alone or together with 0.7mM theophylline [(●–●–●) + epinephrine, (○–○–○) + glucagon] were added. Each point represents the mean value of at least two different experiments. Bars give the SEM of 3 to 5 different experiments. Control values without added hormone but with 0.7 mM theophylline have been subtracted.

## Results and discussion

The smallest amount of cAMP detectable by this assay lies between 0.2 and 0.3 pmoles. In the absence of hormones cAMP concentrations in the medium were about 20 pmoles/g adipose tissue throughout the 1 hr incubation. Epinephrine (0.5 μg/ml) and glucagon (5 μg/ml) did not induce a significant cAMP release (fig. 1), although lipolysis was maximally stimulated under these conditions as indicated by the rates of glycerol release. In contrast, as little as 0.035 μg/ml of glucagon in the perfusion medium caused a large and continuous cAMP release in perfused rat livers [10], whereas epinephrine had a very small effect on cAMP release during liver perfusion [9,10].

When the phosphodiesterase inhibitor theophylline (0.7 mM) was added together with epinephrine or glucagon, a significant release of cAMP into the medium was detected already after 2 minutes (fig. 1 and 2). cAMP continued to accumulate in the medium in a nearly linear fashion during the first 20 min (fig. 1 and 2). After 30 min a maximum was reached (fig. 2) usually in the range of 300 pmoles/g adipose tissue, but sometimes amounting up to 600 pmoles/g adipose tissue. Thereafter, the concentration of the cyclic nucleotide in the medium began to decrease. At 60 min more than 50% had again disappeared (fig. 2A and 2B). Added $^3$H-labelled and cold cAMP ($3.2 \times 10^{-7}$ M) was removed by the tissue. This process was much slower in the presence of 0.7 mM theophyllin (fig. 3A and 3B). The disappearance of added cold cAMP (protein binding assay) was much faster than that of [$^3$H] from added [$^3$H]cAMP. This difference was due to the appearance of labelled metabolites in the medium, mainly [$^3$H]adenosine and [$^3$H]adenosine-5′-phosphate, as determined by thin-layer chromatography (unpublished results). Thus, disappearance of cAMP was not due to diffusion into the tissue but to degradation.

When epinephrine, 0.5 μg/ml, was added in addition to 0.7 mM theophylline and $3.2 \times 10^{-7}$ M cAMP, the difference between the rates of disappearance of $^3$H-labelled or cold cyclic AMP was much more striking (fig. 3A and 3B): whereas the decrease of radioactivity was slow and similar to that in the presence of theophylline alone, there was a very marked and enhanced disappearance of added cAMP (after 90 min no cAMP could be detected in the medium). It would, therefore, appear that epinephrine somehow led to an increased enzymatic degradation of cAMP to its metabolites. The initial rise of cAMP was smaller with than without added cAMP, indicating that a high concentration of cyclic

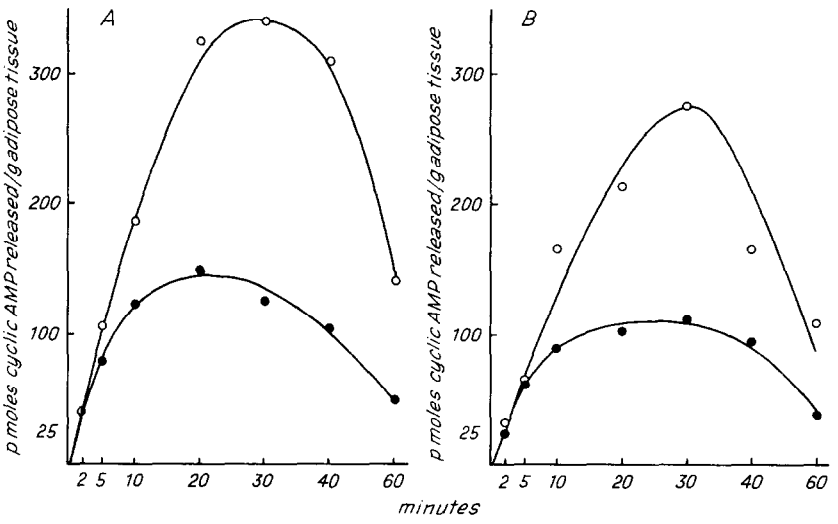


Fig. 2. Effect of insulin (1 mU/ml) and NSILA-S (0.6 mU/ml; specific activity 15 mU/mg protein) on epinephrine-induced (0.5 μg/ml) cAMP release into the medium in the presence of 0.7 mM theophylline. (○–○–○) epinephrine + theophylline; (●–●–●) epinephrine + theophyllin + insulin (A) or NSILA-S (B). Experimental conditions are described in the text. Control values without added hormones but with 0.7 mM theophylline have been subtracted.

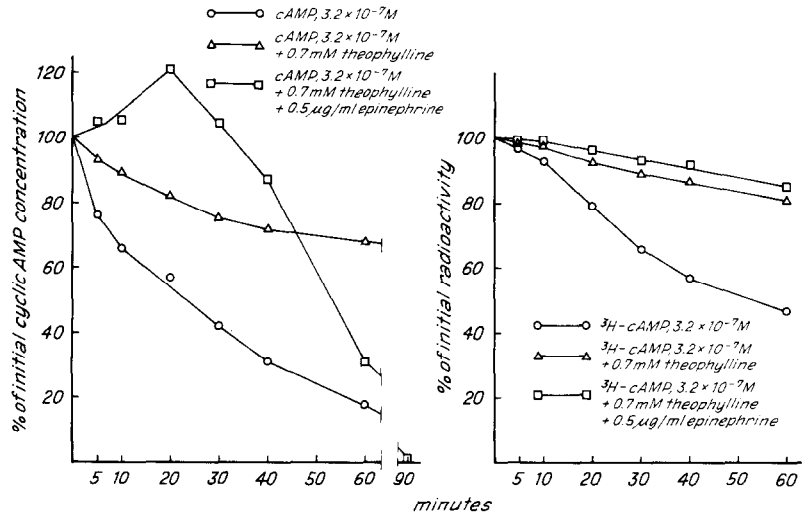


Fig. 3. Disappearance of cAMP from the medium in the presence of adipose tissue. Cold cAMP ($3.2 \times 10^{-7}$ M, fig. 3A) or [$^3$H]cAMP ($3.2 \times 10^{-7}$ M, fig. 3B) without theophylline (○–○–○), with 0.7 mM theophylline (△–△–△) or with 0.7 mM theophylline and 0.5 μg/ml epinephrine (□–□–□) were added to the incubation vials after 10 min of preincubation. Radioactivity was counted after different time intervals; cold cAMP was determined by the protein binding assay. Each point gives the mean of two different experiments.

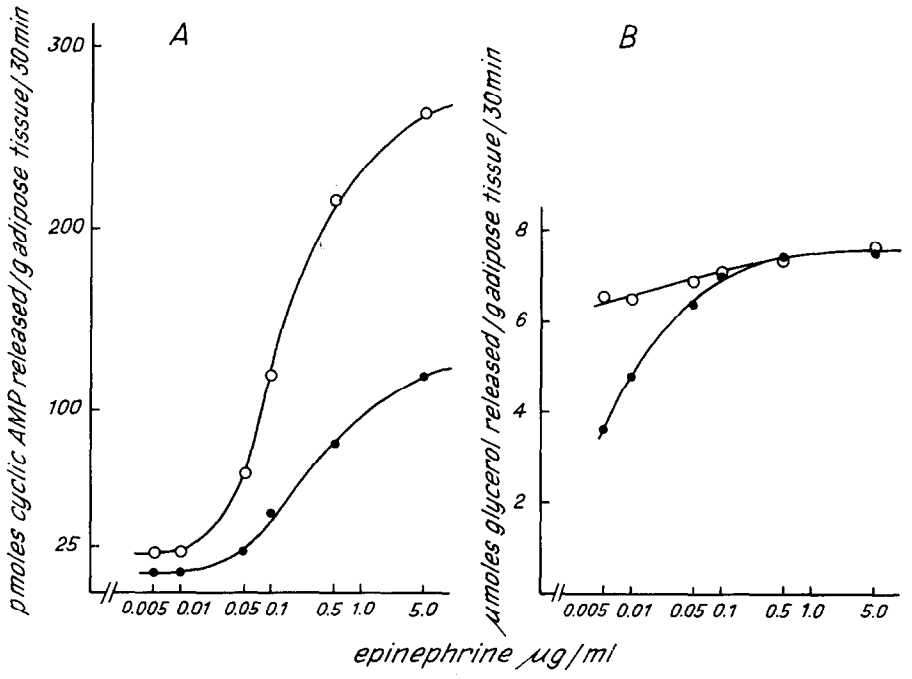


Fig. 4. Dose-response curves of epinephrine-induced cAMP (A) and glycerol release (B) in the presence (•–•–•) and absence (o–o–o) of 1 mU/ml of insulin. Incubations were carried out for 30 min. Each point represents the mean value of two incubations.

nucleotide in the medium inhibits its hormone-induced release from the tissue. Hence, these results provide indirect evidence that in a second phase, after initial adenylcyclase activation by epinephrine in the presence of theophylline, either 1) adenylcyclase activity is inhibited and cAMP degraded by the remaining phosphodiesterase activity not completely suppressed by theophylline and/or 2) phosphodiesterase activity is stimulated. Both possibilities could be regarded as reasonable feedback mechanisms to prevent overactivation of cAMP-dependent processes within the cell.

The effect of glucagon (5 μg/ml) on cAMP release was smaller than that of epinephrine (fig. 1). This is in agreement with the finding of Butcher et al. [4] according to which intracellular cAMP levels in isolated fat cells maximally stimulated with glucagon and caffeine were only 50% of those obtained with epinephrine and caffeine.

If intracellular cAMP levels in adipose tissue following epinephrine stimulation (1 μg/ml) in the presence of caffeine (1 mM) are in the order of 1 nmole/g as reported by Butcher et al. [3], one fourth to one half of the intracellular cAMP which accumulates during hormone action is released into the medium within 30 min. In Butcher's experiments, the intracellular cAMP concentration reached a maximum between 5 and 10 min after stimulation and then remained constant for another 10 min. Thus, during the first 5 min after hormone addition cAMP accumulates primarily within the cell, whereas an equivalent of the entire amount of the nucleotide formed between 10 and 30 min is released into the incubation medium. In addition, some cAMP may be destroyed by the remaining phosphodiesterase activity.

With theophylline alone, there was an immediate and variable cAMP release into the medium (between 50 and 150 pmoles/g adipose tissue). The cAMP concentration in the medium remained constant during the entire incubation period (not shown).

The effects of insulin and NSILA-S on epinephrine-induced cAMP release in the presence of 0.7 mM theophylline are shown in fig. 2A and 2B. Both compounds

in concentrations of 1 mU/ml and 0.6 mU/ml, respectively, reduced the release of cAMP to less than 50%. Similar effects of insulin have been reported on intracellular cAMP levels in intact adipose tissue [3] and in isolated fat cells [4,5]. Our results strengthen the already available evidence that insulin and NSILA-S act on adipose tissue by the same molecular mechanism.

The effect of different concentrations of epinephrine on cAMP and glycerol release in the presence of theophylline and insulin (1 mU/ml) is shown in fig. 4A and 4B. 0.05 μg/ml of epinephrine caused a marked cAMP release; 5 μg/ml seemed to exert a submaximal effect. However, stimulation of glycerol release was nearly maximal with as little as 0.005 μg/ml of epinephrine. Insulin reduced cAMP release at all epinephrine concentrations, whereas it reduced glycerol production only at epinephrine concentrations below 0.1 μg/ml. At higher epinephrine concentrations the effect of insulin on glycerol release was no longer seen. Although some correlation between cAMP release and lipolysis is suggested under certain conditions, the quantitative relationship between the two is not clear. Nevertheless, cAMP release in the presence of phosphodiesterase inhibitors may serve as a simple model to demonstrate hormone-induced modulation of 'free', metabolically available cAMP in intact adipose tissue.

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