cAMP INDEPENDENT STIMULATION OF LIPOLYSIS IN ISOLATED FAT CELLS?*

Ursula LANG and Robert SCHWYZER
Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, CH-8049 Zürich, Switzerland

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1. Introduction

It is well established that effects of polypeptide hormones, including ACTH, on their target cells are mediated by *c*AMP produced from ATP by membrane cyclase [1]. Whether or not *c*AMP is an *obligate* second messenger is, however, not clear. Our studies with a potent ACTH derivative, (N^{ϵ}-dansyllysine^{21})-ACTH-(1–24)-tetrakosipeptide [2], suggest that *c*AMP might be responsible for only a part (quantitatively and/or qualitatively) of the effects in adrenal and fat cells, and that the peptide hormone might well be capable of stimulating steroidogenesis and lipolysis by other mechanisms as well. This would satisfy the cybernetic postulate of achieving optimal functional integrity of a vital regulatory cycle by redundancy, that is by providing more than one link between (hormonal) signal and effect.

The results presented here show that actinomycin D can at the same time *enhance* the effect of ACTH on lipolysis in isolated fat cells and *suppress* the increase of *c*AMP in response to this hormone. We suggest that this is further evidence in favor of our working hypothesis that *c*AMP is not necessarily involved in mediating all of the responses to ACTH, at least not in these target cells.

2. Experimental

Fat cells were prepared from epididymal fat pads of rats weighing 180–200 g according to Rodbell [3]. Incubation with collagenase was carried out at 37° in Krebs-Ringer phosphate medium containing 3.5% human serum albumin for 1 hr. After passage through a silk screen the cells were washed twice with fresh medium at 37°. In each experiment, 0.5 ml of fat cells from a single pool was incubated in 1.5 ml of the Krebs-Ringer medium containing 10 nmoles of ATP. Two aliquots were used for the determination of *c*AMP and glycerol, one for dried cell weights (15 hr at 65°).

Glycerol was determined enzymatically according to Wieland [4] and calculated as nmoles per mg dried cells.

*c*AMP was assayed with a luciferine–luciferase technique [5] modified as follows:
1) Destruction of unwanted adenylates: A 0.5 ml aliquot of the incubated cell suspension was added to 2.5 ml of glycine buffer in a boiling water bath (0.1 M glycine adjusted to pH 7.5 with 0.1 N NaOH) and heated for 10 min. 400 µl of the denatured solution were then incubated for 4 hr at 37° with 100 µl of a 20 mM $MgCl_2$, 200 mM Tris-HCl buffer at pH 7.5 containing 0.15 mg/ml alkaline phosphatase and

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Abbreviations:
ACTH = adrenocorticotrophic hormone; *c*AMP = *cyclo*-3′,5′-adenosine monophosphate; ATP and AMP = adenosine-5′-tri- and -monophosphate, respectively; GTP and GDP = guanosine-5′-tri- and -diphosphate, respectively; Tris-HCl = trishydroxymethyl-amine hydrochloride. Other abbreviations according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature, European J. Biochem. 1 (1967) 375.

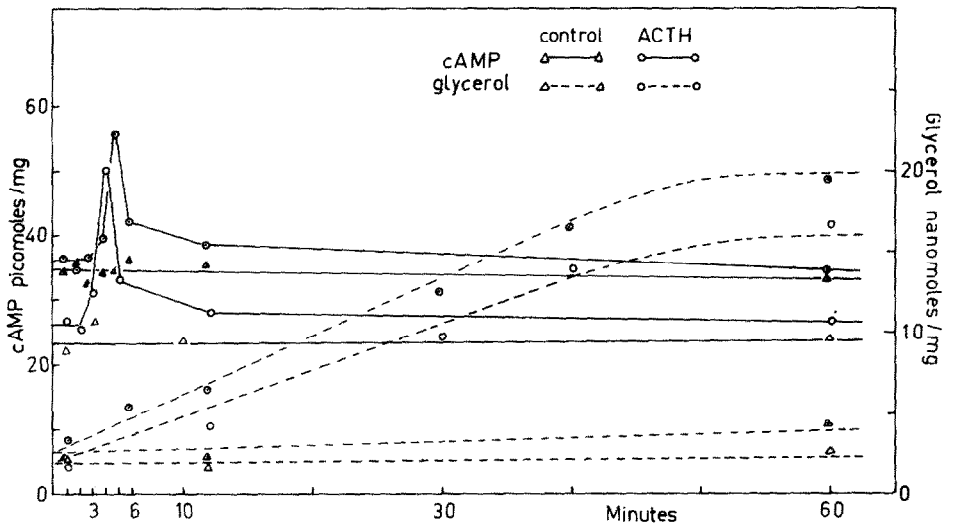

Fig. 1. Effect of 1.25 μg/ml (≃ 3.5 × 10^{-8} M) ACTH on *c*AMP levels and on lipolysis in isolated rat fat cells (2 experiments each with different cell batches).

0.7 mg/ml apyrase (Sigma Chemicals). The alkaline phosphatase was then destroyed by heating at 100° for 2 hr.

2) 5′-AMP from *c*AMP: The resulting solution containing *c*AMP as the only adenylate was incubated for 30 min at 37° with 50 μl of a 100 mM KCl, 6 mM $MgSO_4$ solution containing 2 mg/ml 3′,5′-cyclonucleotide phosphodiesterase (Sigma Chemicals). The enzyme was then inactivated by heating for 2 min at 100°.

3) ATP from 5′-AMP: To the above solution were added 200 μl of the following kinase mixture: 6 mM $MgSO_4$, 100 mM KCl, 0.1 mM phosphoenol pyruvate, 10 nM ATP, 50 μg/ml myokinase and 75 μg/ml pyruvate kinase (both from Boehringer GmbH). The tubes were covered and incubated at 37° for 90 min.

4) Luciferine–luciferase reaction: The resulting ATP-containing solutions were poured into 10 ml of a 100 mM sodium arsenate, 20 mM $MgSO_4$ buffer at pH 7.5, containing 30 mg/100 ml of a crude firefly luciferin–luciferase system (Sigma Chemicals). The number of photons produced per time unit was measured with a liquid scintillation counter (Nuclear-Chicago Mark 1, Model 7008). ATP (≡ *c*AMP) was calculated as picomoles/mg of dried cells.

ACTH was ACTH-(1–24)-tetrakosipeptide [6]. Actinomycin D and adrenaline (epinephrine) were purchased from Sigma Chemicals.

The hormones were added at time 0, the antibiotic at time 0 or −15 min (without changing the results significantly).

3. Results and discussion

Stimulation of isolated rat fat cells by ACTH *without* diesterase inhibition by methyl xanthines produces an initial "burst" of total *c*AMP which reaches its maximum about 3–5 min after addition of the hormone (fig. 1). Thereafter, the *c*AMP concentration rapidly decreases to control values, despite continued presence of the hormone*. In contrast to *c*AMP production, lipolysis immediately** proceeds at a rather steady, enhanced rate for about 1 to 1½ hr.

The question arises as to the relation between the time-restricted enhancement of *c*AMP and the long term acceleration of lipolysis: is there a direct stimu-

* Our results agree with those of Manganiello et al. [10] except that we found the absolute levels of *c*AMP to be higher (an as yet unexplained difference which might have something to do with the strain of rats or with the cell preparation).

** Because of the very rapid onset of both events, it wasn't possible (with the methods used) to decide whether or not *c*AMP production precedes enhanced lipolysis.

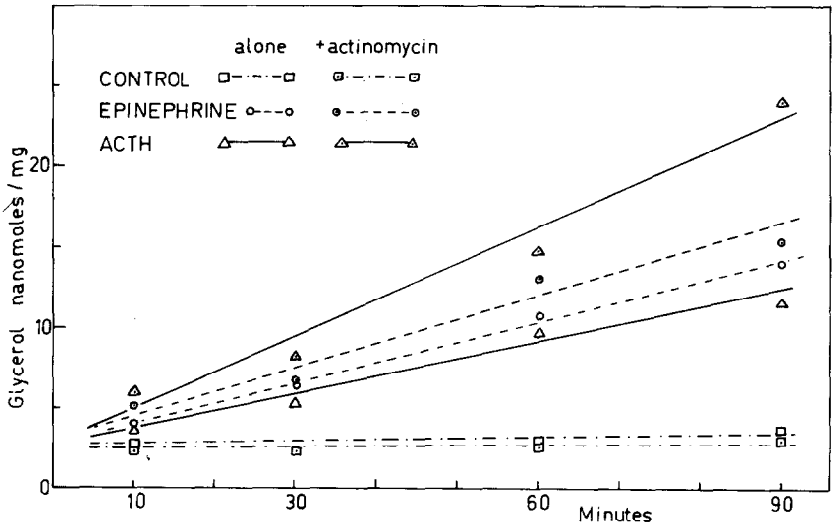

Fig. 2. Effect of actinomycin D (5×10^{-6} M) on ACTH (1.25 μg/ml) and adrenaline (1 μg/ml) stimulation of lipolysis in isolated rat fat cells. Preincubation with actinomycin was for 15 min.

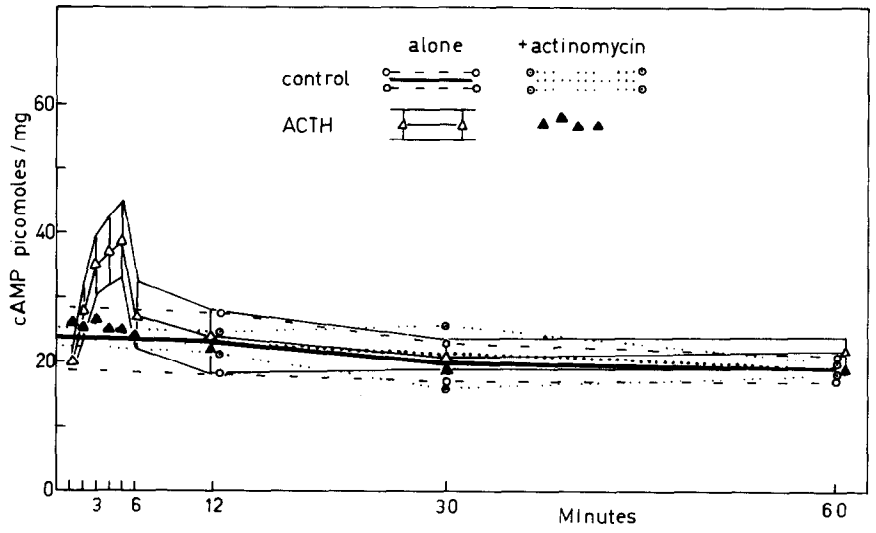

Fig. 3. Effect of actinomycin D (5×10^{-6} M) on ACTH (1.25 μg/ml) stimulation of *c*AMP production by isolated rat fat cells (means and standard deviations of 6 experiments). Preincubation with actinomycin was for 15 min.

lation of preformed lipolytic enzymes (either allosterically, by phosphorylation, or by release), or does *c*AMP cause de novo synthesis? Although the immediate onset of lipolysis seems to preclude a mechanism based solely on enzyme synthesis, we decided to test actinomycin (which is known to inhibit the transcription of structure genes) to see whether or not a synthetic component is involved in the hormonal effect. *Inhibition* of lypolysis would indicate the possibility of protein synthesis as a link of events, the observation of *no effect* would probably rule it out.

Fig. 2 shows the results with an actinomycin D concentration which usually inhibits transcription by 90%. Instead of blocking the ACTH effect or leaving it unchanged, the rate of lipolysis is *enhanced* about 2-fold (chloramphenicol, cycloheximide, and puromycin had no effect). Contrastingly, the same concentrations of actinomycin *inhibit* the appearance of the typical *c*AMP burst completely, without, however, changing the control values (fig. 3).

We cannot yet explain these unexpected observations. A number of possible causes come to mind, i.e. complexation by actinomycin D [7] of guanosine-5′-phosphates, GTP and GDP. These are known to be specific and obligatory agents linking stimulus with response, i.e. the process of glucagon receptor interaction with that of adenyl cyclase activation in rat liver plasma membranes [8]. They are also supposed to decrease the uptake of glucagon by the same membranes [9]. A lowering of guanyl nucleotide activity (concentration) by complexation would hence be expected to inhibit the *c*AMP response to ACTH and to increase the chance of ACTH reaching and activating other receptors, *provided* that the same conditions prevail in the ACTH–GTP(GDP)–rat fat cell preparation as in the glucagon–GTP(GDP)–rat liver membrane system. These other hypothetical receptors would then be responsible for the observed activation of lypolysis. We are experimentally testing this and other working hypotheses.

Preliminary experiments with adrenaline (epinephrine) show that actinomycin D produces only a very slight effect on both lipolysis and *c*AMP "burst", indicating a rather pronounced hormonal specificity.

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