INHIBITION BY ACTINOMYCIN D, CYCLOHEXIMIDE AND PUROMYCIN OF STEROID SYNTHESIS INDUCED BY CYCLIC AMP IN INTERSTITIAL CELLS

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SUMMARY

Adenosine 3'5'-cyclic monophosphate induces de novo steroid synthesis in a clonal epithelial culture line, derived from a mouse testicular interstitial cell tumor. Actinomycin D, cycloheximide and puromycin each completely blocks the action of the inducer, indicating that transcription and translation may be required for the induction.

The role of adenosine 3'5'-cyclic monophosphate (cAMP) as the common intracellular mediator of a number of hormones and biogenic amines is well established (1). Gill and Garren demonstrated recently that a cAMP-dependent protein-phosphokinase from adrenal cortex tissue contains a cAMP-receptor site which regulates the catalytic activity (2), showing that the cyclic nucleotide acts as an activator of a specific enzyme as has been postulated (3). In addition to its role as a physiological modulator in the higher animals, cAMP has also been shown to control various cellular processes in non-vertebrate organisms. For example, there exists a protein which binds cAMP with high affinity and which is required for the full transcriptional activity in a cell free system of E. coli (4). In this case at least, cAMP is a crucial regulator substance for mRNA synthesis. That the steroidogenic actions of adrenocorticotropic hormone

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(ACTH) and interstitial cell stimulating hormone (ICSH or LH) both utilize cAMP as the common intracellular "messenger" has been well documented (5,6), but whether the action of cAMP itself in the steroid-secreting tissues involves the regulation of transcription or whether cAMP merely activates one or more enzymes which catalyze the rate-limiting steps in steroid synthesis is still not known. Here we report on the properties of a clonal, established cell line of the mouse testicular interstitial (Leydig) cells that bear on this question.

The establishment of the original culture line from a transplantable Leydig cell tumor in a Balb/cJ mouse, the conditions of cell culture and methods of steroid assay have been reported earlier (7). For the present study, we employed a clonal subline, designated I10A, which is characterized by a very low basal level of steroid synthesis coupled with a high sensitivity to induction by cAMP. As in the original mass culture from which it was derived, this clone upon induction secretes two steroid products, which are accumulated in the culture medium at high concentrations. The steroids are 20 α-hydroxypregn-4-en-3-one and progesterone. Since both absorb light very strongly at 240 nm, the steroid synthetic activity of the culture is routinely assayed spectrophotometrically (7). (In the following figures and tables, an O.D. value of 1.00 corresponds to 18.7 μg of total steroids.)

The main characteristics of the cAMP-induced steroid synthesis in clone I10A can be summarized as follows: a) The lowest effective level of cAMP at which significant induction can be observed is 5×10^{-6} M. This compares favorably with the physiological concentrations of cAMP in the bovine adrenal cortex *in situ*, which is in the range of $0.5 - 5 \times 10^{-6}$ M (8). Maximal induction requires 10^{-4} M cAMP. b) Induction of steroid synthesis shows no measurable lag time and continues linearly for at least 20 hrs. c) The presence of the inducer in the culture medium is necessary for the continued synthesis of steroid. When the inducer is removed from the culture medium,

synthesis returns immediately to the basal level. The effect of metabolic inhibitors on cAMP-induced steroid synthesis is described below.

Effect of Actinomycin D: The actual level of inhibition of the cellular RNA synthesis by actinomycin D was first determined by pulse labelling the cultures for 30 mins with uridine-5-^{3}H. Incorporation of the tritiated precursor into the total RNA fraction was measured according to Kahan (9). It was found that the inhibition of the total cellular RNA synthesis by actinomycin D in this culture was a gradual dose-dependent process, requiring no lag time. Under the normal culture conditions, the inhibition was 16.0 % at 0.01 μg actinomycin D/ ml, 51.4 % at 0.1 μg/ ml and 87.2 % at 1.0 μg/ ml. At actinomycin D concentration of 4.0 μg/ ml, the inhibition was virtually complete (>98 %). The inhibition of RNA synthesis is accompanied by a parallel inhibition of the steroid synthesis induced by cAMP, as shown in Fig. 1.

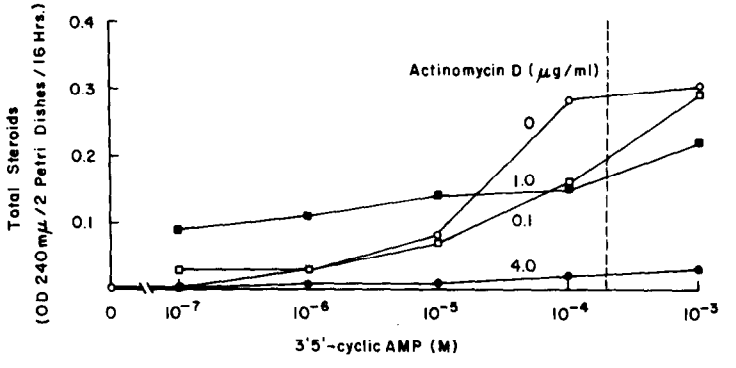

Fig. 1. Dose-dependent inhibition by actinomycin D of steroid synthesis induced by increasing doses of cAMP.

Without actinomycin D, the standard steroid induction curve is obtained, the maximum being reached with 10^{-4} M cAMP (Fig. 1, at 0 μg/ml). At 4.0 μg/ ml, actinomycin D blocks this induction completely. The fact that an intermediate level of the inhibitor (1.0 μg/ml), which by itself reduces the cullular RNA synthesis by 90 %, actually stimulates the steroid synthesis slightly if the cAMP concentration is sub-optimal, is surprising and remains unexplained.

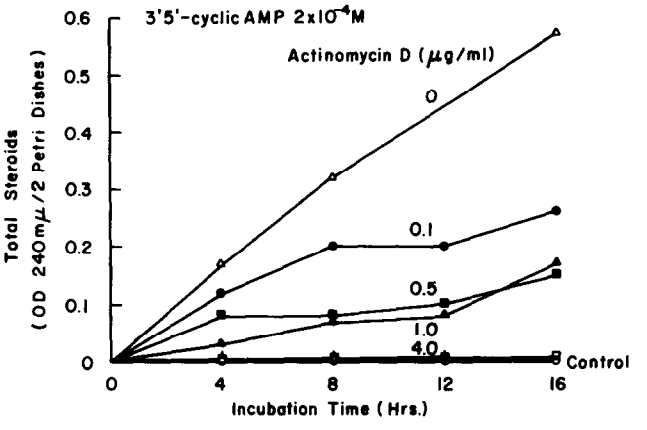

Fig. 2. Inhibition by actinomycin D of steroid synthesis at a saturating concentration of the inducer. Time course.

A time-course experiment, in which 2 X 10^{-4} M cAMP was used (indicated by the vertical broken line in Fig. 1), is summarized in Fig. 2. At this saturating level of cAMP, the inhibitory effect of actinomycin D predominates at all concentrations up to 16 hrs. Pre-exposure of the cultures to the inhibitor also

Table 1. Effect of Pretreatment with Actinomycin D

On Subsequent Induction with cAMP

Replicate culture plates (60 X 15 cm Petri dishes) were first incubated in paired groups with and without actinomycin D for 16 hrs. The plates were then washed twice with fresh culture medium and incubated further for 12 hrs with 20 mM cAMP minus actinomycin D. Steroid values are from 2 Petri dishes.

Group No.	Pretreatment for 16 hrs with: Act. D (μg/ml)	Pretreatment for 16 hrs with: cAMP (M)	Total steroid secreted in next 12 hrs with cAMP ($O.D._{240nm}$)
1	0.0	0.0	0.46
2	0.0	10^{-3}	0.26
3	0.1	10^{-3}	0.04
4	1.0	10^{-3}	0.05
5	4.0	10^{-3}	0.04

blocks the subsequent induction by cAMP, indicating that the recovery to inducibility by the cell cannot be effected by simply withdrawing the anti-metabolite from the medium (Table 1).

Effect of Cycloheximide and Puromycin: Data given in Table 2 demonstrate that both puromycin and cycloheximide, at concentrations which inhibit the total cellular protein synthesis without acute cytotoxic effects, also inhibit completely the induction of steroid synthesis by cAMP.

Table 2. Effect of Cycloheximide and Puromycin On Steroid Synthesis Induced by cAMP

Group No.	cAMP (M)	Inhibitor (10 μg/ml)	Total steroid secreted in 16 hrs ($O.D._{240nm}$)
1	0.0	None	0.04
2	2×10^{-4}	None	1.10
3	2×10^{-4}	Cycloheximide	0.04
4	2×10^{-4}	Puromycin	<0.15

It had been reported earlier that the stimulatory action of ACTH on adrenal cortex tissue is inhibited by puromycin or chloramphenicol (10, 11). This was found to be true also with the adrenal cortex cell cultures derived from a mouse adrenal tumor (12). Since cAMP is the common mediator involved in both the ACTH- and the ICSH-dependent steroid synthesis, it was of interest to test if the induction of steroidogenesis in the interstitial cell culture also involved step(s) sensitive to the inhibitors of protein and RNA syntheses. The main advantage of the present culture system for such a study is its extreme sensitivity towards cAMP as well as the ease of experimentation. In contrast, incubating slices of corpora lutea (13) or rat testis tissue (14) required exogenous cAMP in excess of 2×10^{-2} M before significant stimulation of steroid

synthesis could be demonstrated. The maximal intracellular concentration of cAMP in the adrenal cortex immediately after stimulation with ACTH is only 5×10^{-6} M (8), which falls within the range of cAMP concentrations with which the clone I10A can be induced.

The results presented here strongly suggest that at least in the interstitial cell, the induction of steroid synthesis by cAMP (and presumably also by ICSH) may require the synthesis not only of new proteins but also of short-lived mRNA molecules to code for them.

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