METABOLIC REGULATION OF STEROIDOGENESIS IN ISOLATED ADRENAL CELLS OF RAT. EFFECT OF ACTINOMYCIN D ON cGMP-INDUCED STEROIDOGENESIS.

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Summary: The data presented with the isolated adrenal cells in the present study show that guanosine cyclic 3',5'-monophosphate (cGMP)-stimulated steroidogenesis is actinomycin-sensitive. cGMP does not have any effect on the synthesis of corticosterone from (20S)-20-hydroxycholesterol and pregnenolone. This suggests that the conversion of cholesterol to (20S)-20-hydroxycholesterol is the step at which transcriptional control by cGMP is manifested. Previously, it has been inferred that the transformation of cholesterol to (20S)-20-hydroxy-cholesterol is the rate-limiting cycloheximide-sensitive step which is stimulated by ACTH and cAMP.

It is, therefore, suggested that the action of ACTH in stimulating corticosterone synthesis is dependent on both cGMP and cAMP. It is proposed that cGMP may be responsible for the formation or processing of specific mRNA which in turn is then translated by means of a cGMP or a cAMP-dependent phosphorylation factor (s).

Introduction

The availability of the isolated adrenal cell system (1-3), which is highly responsive to physiological concentrations of ACTH[1], and the isolated adrenocortical carcinoma and cultured cell preparations (4,5) in which ACTH action in controlling steroid synthesis is highly modified (4-10), made it possible (11,12) to demonstrate that cAMP is not the sole mediator of ACTH in the control of adrenal steroid-

Trivial Names and Abbreviations

1. The trivial names and abbreviations used are: ACTH, adrenocorticotropic hormone; cAMP, adenosine cyclic 3':5'-monophosphate; cGMP, guanosine cyclic 3':5'-monophosphate; pregnenolone, 5-pregnen-3β-ol-20-one; corticosterone, 11β,21-dihydroxy-4-pregnen-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnen-3,20-dione.

ogenesis (11,12). Recently the evidence was provided that ACTH-stimulated steroidogenesis is mediated by both cGMP and cAMP, the former nucleotide mediating the ACTH control of corticosterone synthesis at low concentration of the hormone and the latter at higher concentration (13,14).

The present studies were designed to further elucidate the mode of control of cGMP-induced steroidogenesis. In this manuscript we report a unique actinomycin-sensitive effect of cGMP.

Materials and Methods

The isolated adrenal cells were prepared by trypsin digestion (1-3). The method of incubation for ACTH, cGMP, or other appropriate agent, was that already described (2,3). In general for each isolated adrenal cell preparation, adrenals from 16 rats were used, and the cells from each adrenal (approximately 2 X 10^6 cells) were resuspended in 0.8 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 4% albumin and 0.2% glucose. Corticosterone was measured fluorometrically (15).

The method of incubation with double labeled radioactive pregnenolone was as previously described (6): incubation was carried out in Teflon Flasks. Each flask contained 20 ml isolated adrenal cell suspension (2,3) obtained from the adrenals of 16 rats. In addition to the appropriate amount of cell suspension, the first flask contained a mixture of 20 µCi of [7α-^{3}H]pregnenolone (specific activity 5Ci/mmol) and 1 µCi of [4-^{14}C]pregnenolone (specific activity 50 mCi/mmol) (^{3}H/^{14}C ratio 20.00); the second flask contained 20 µCi of [7α-^{3}H] pregnenolone + cGMP (10 mM) and the third flask contained 1 µCi of [4-^{14}C]pregnenolone. The incubation was continued for 2.5 h and the reaction was stopped by the addition of 15 ml of distilled water and 75 ml of methylene chloride into each flask. The contents of the

second flask which contained [7α-^{3}H]pregnenolone + cGMP were mixed with the contents of the third flask which contained [14-^{14}C]pregnenolone. To the reaction mixture of the two flasks 15 mg of deoxycorticosterone and 15 mg of corticosterone were added and the products processed identically.

The experiments involving cGMP with (20S)-20-hydroxy[7α-^{3}H] cholesterol were conducted as previously described (11). Each flask contained 20 ml of suspended isolated adrenal cells (2,3) obtained from the adrenals of 16 rats. In addition to the appropriate amount of suspended cells, each flask contained 1.60 μCi of (20S)-20-hydroxy [7α-^{3}H]cholesterol with and without 10 mM of cGMP. The incubation was carried out for 2.5 h and the reaction mixtures processed as mentioned previously.

The isolation of deoxycorticosterone and corticosterone was carried out on the above residues by various thin-layer chromatographic operations as previously described (7,11). The isolated deoxycorticosterone was acetylated (7,16), further purified by thin-layer chromatography (7,11) and crystallized from acetone-n-hexane to constant specific activity. The purified corticosterone was crystallized to constant specific activity from acetone-ligroin.

Measurement of [^{14}C]leucine incorporation into protein synthesis was as follows: [^{14}C]leucine (0.25 μCi) was incubated with 1 ml of isolated adrenal cell suspension (2,3) containing cGMP (10 mM) or cGMP + actinomycin (2 μM) for 2 h. Cells were cooled on ice, centrifuged (200 g; 10 min) and the supernatant discarded. All procedures were performed at 0°. The cells were washed with 0.9% NaCl. Total protein was precipitated by the addition of 3 ml, 5% (w/v) trichloroacetic acid. The samples were recentrifuged (200 g; 10 min) and the precipitate was treated with 5% trichloroacetic acid (two times 3 ml) & finally washed with 3 ml of 95% ethanol. The final residue was

dried and dissolved in 1 ml of NCS Tissue Solubilizer; 10 ml of Aquasol was added and the samples were counted for radioactivity in this acid-precipitated fraction.

ACTH, a United States Pharmacopeia Standard, was purchased from United States Pharmacopeia. All other chemicals were reagent grade and were obtained commercially.

Results

Effect of Cycloheximide and Actinomycin D on cGMP-Stimulated Steroidogenesis - Cycloheximide, but not actinomycin D, is known to inhibit steroidogenic response of ACTH in vivo (17), in vitro quartered adrenal gland (18) and in an isolated adrenal cell (2,7). In an attempt to evaluate the effect of these inhibitors on cGMP-stimulated steroidogenesis in an isolated adrenal cell, 2 μM and 10 μM concentration of actinomycin or 10 μM concentration of cycloheximide was used with 8 mM and 10 mM cGMP. Previously, it was shown that the above concentrations of the cyclic nuleotide stimulated approximately half and near maximum synthesis of corticosterone, respectively (2). The results in Table I show that actinomycin at both concentrations inhibited steroidogenesis ($^{a}p=0.001$ & $^{b}p< 0.001$). Furthermore, the incubation of actinomycin with cGMP and pregnenolone inhibited the corticosteroidogenesis stimulated by cGMP ($^{c}p< 0.005$) but had no effect on the transformation of pregnenolone to corticosterone. This would be in keeping with the concept that cGMP, similar to ACTH and cAMP, stimulates corticosterone synthesis from endogenous cholesterol present in the adrenal cell (1,2). These results, therefore, suggest that the steroidogenic step stimulated by cGMP lies before the formation of pregnenolone.

In an analogous manner to the results obtained with ACTH and cAMP (2,7,11), cycloheximide inhibited, almost completely, the cGMP-stimulated endogenous synthesis of corticosterone (Table I).

Effect of cGMP on the Transformation of Pregnenolone to Deoxycorti-

Table I

Effect of cycloheximide and actinomycin D on cGMP-stimulated steroidogenesis in isolated adrenal cell preparation.

Incubation system: adrenal cell suspension, 0.8 ml; reagents dissolved in 0.2 ml vehicle. Total volume of incubation 1 ml. The values shown are derived from at least twelve observations and are given as means ± standard deviation. Control value has been subtracted from the experimental results. Values of p were calculated by the student t test.

Additions	Corticosterone
	µg/2 hrs
Control	0.051 ± 0.010
ACTH (100 microunits/ml)	2.230 ± 0.025
cGMP (8 mM)	0.755 ± 0.037[a]
cGMP (10 mM)	1.293 ± 0.051[b]
Pregnenolone (32 µM)	2.328 ± 0.104
Cycloheximide (10 µM)	0.020
Actinomycin D (2 µM)	0.002
Actinomycin D (10 µM)	0.012
Pregnenolone + Actinomycin D (10 µM)	2.164 ± 0.100
Pregnenolone + Cycloheximide	2.057 ± 0.092
cGMP (8 mM) + Cycloheximide	0.131 ± 0.038
cGMP (10 mM) + Cycloheximide	0.046 ± 0.021
cGMP (10 mM) + Pregnenolone	4.169 ± 0.251[c]
cGMP (10 mM) + Pregnenolone + Cycloheximide	2.402 ± 0.271
cGMP (10 mM) + Actinomycin D (2 µM)	0.688 ± 0.030[b]
cGMP (8 mM) + Actinomycin D (10 µM)	0.437 ± 0.058[a]
cGMP (10mM) + Actinomycin D (10 µM)	0.748 ± 0.080[b*]
cGMP (10 mM) + Pregnenolone + Actinomycin D (10 µM)	2.881 ± 0.211[c]

[a] $p=0.001$ $(n=12)$; [b] $p<0.001$; [b*] $p<0.001$ $(n=14)$; [c] $p<0.005$

Table II

Effect of cGMP on the transformation of [^{3}H] pregnenolone into deoxycorticosterone and corticosterone in isolated adrenal cells.

The [^{3}H/^{14}C] ratios of the products (and their derivatives) obtained after the incubation of [4-^{14}C, 7α-^{3}H]pregnenolone with isolated adrenal cells. Incubation was carried out in 3 flasks containing 20 ml isolated adrenal cell preparation as mentioned in text. Flask 1 contained a mixture of [7α-^{3}H]pregnenolone (20 μCi) + [4-^{14}C]pregnenolone (1 μCi) (^{3}H/^{14}C ratio 20.00); flask 2 contained [7α-^{3}H]pregnenolone (20 μCi) + cGMP (10mM) and the flask 3 contained [4-^{14}C]pregnenolone (1 μCi). The incubation was for 2.5 h and the reaction was stopped by the addition of 75 ml methylene chloride to each flask. The contents of the second and third flasks were mixed and deoxycorticosterone and corticosterone isolated as described in "Text".

Compound	Crystallization	[^{3}H/^{14}C] ratio of compound from Flask 1	Flask 2+Flask 3
Deoxycorticosterone acetate	Crude Product	15.77	16.60
	1st	15.65	16.48
Corticosterone	1st	19.53	17.22
	2nd	17.20	17.38

Costerone and Corticosterone - Studies carried out in the adrenal cells where one group of cells was incubated with [7α-^{3}H]pregnenolone + cGMP and the other group with [4-^{14}C]pregnenolone and the incubation solutions mixed after terminating the incubation, showed no significant change

Table III

Effect of cGMP on the transformation of (20S)-20-hydroxy[7α-^{3}H] cholesterol into corticosterone in isolated adrenal cell preparation.

The total ^{3}H disintegrations per min of corticosterone obtained after the incubation of (20S)-20-hydroxy[7α-^{3}H] cholesterol with isolated adrenal cells. Incubation was carried out in Teflon flasks containing 20 ml of isolated adrenal cell preparation as mentioned under "Text". Each flask contained (20S)-20-hydroxy [7α-^{3}H]cholesterol (1.60 μCi) and cGMP (10 mM). The incubation was for 2.5 h and the reaction was stopped by the addition of 75 ml of methylene chloride to each flask. Corticosterone was isolated as described under "Text".

^{3}H radioactivity of corticosterone from	
Control	+ cGMP
525,000 ± 72,000	439,00 ± 17,000

in the $^{3}H/^{14}C$ ratios of the stimulated cells from that noted in the non-stimulated cells. This confirms the results mentioned in the previous section that cGMP has no effect on the conversion of pregnenolone to corticosterone.

Effect of cGMP on the Transformation of (20S)-20-hydroxycholesterol to Corticosterone - In order to designate the step at which transcriptional control of cGMP is manifested, studies were conducted to examine the effect of cGMP on the conversion of (20S)-20-hydroxycholesterol to corticosterone. cGMP was incubated with (20S)-20-hydroxy[7α-^{3}H]

cholesterol in the isolated adrenal cells. The results in Table III show that cGMP does not have any significant effect on the synthesis of corticosterone from (20S)-20-hydroxycholesterol.

Time Course of the Inhibitory Effect of Actinomycin D on cGMP-Stimulated Steroidogenesis - To study the time relationship of the inhibition of cGMP-stimulated corticosterone synthesis by actinomycin D, corticosteroidogenesis was measured from 0 to 120 minutes. Fig. I shows that only after 60 minutes does the inhibition by actinomycin D become significant. This would imply that the action of cGMP in the

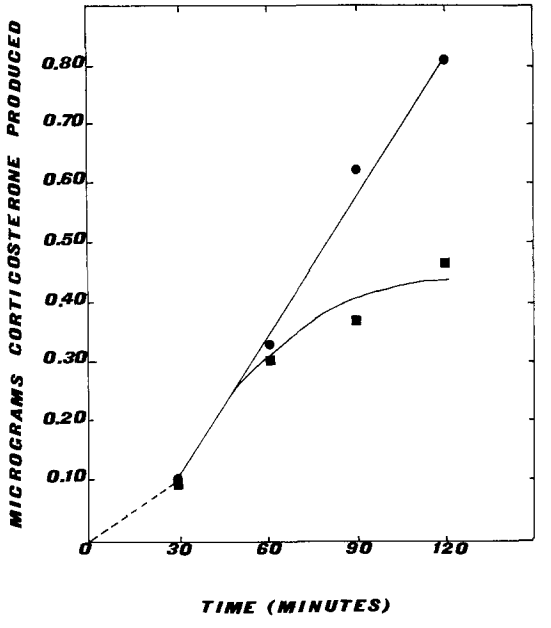

Figure I. Time Course of the Inhibition of cGMP-Induced Corticosterone Synthesis with Actinomycin D. cGMP, ●—● ; cGMP + Actinomycin D ■—■ ; the conditions of the experiments are similar to Table I. Means of triplicate observations are given.

Table IV

Effect of Actinomycin D on Protein Synthesis

[^{14}C]leucine (0.25 μCi) was incubated with 1 ml of isolated adrenal cell suspension (2.0 X 10^6 cells, representing one adrenal gland) containing cGMP (10 mM) or cGMP + actinomycin D (2 μM) for 2 h. Cells were cooled on ice, centrifuged and the trichloroacetic acid fraction obtained as described in "Text". The standard error of the mean for the incorporated radioactivity is given. Results are expressed as [^{14}C]cpm of leucine incorporated into trichloroacetic acid precipitable fraction.

Additions	^{14}C cpm incorporated into trichloroacetic acid precipitable fraction
[^{14}C]leucine + cGMP	8,082 ± 456
[^{14}C]leucine + actinomycin D	8,162 ± 87
[^{14}C]leucine + cGMP + actinomycin D	7,863 ± 704

transcriptional control process is quite rapid.

Effect of Actinomycin D on Protein Synthesis - In order to rule out the possibility that the levels of, actinomycin D, used in this investigation might alter translation as well as transcription and thereby inhibit protein synthesis, the effect of the inhibitor on the incorporation of [^{14}C]leucine into protein synthesis was measured in the presence and absence of cGMP. Table IV shows that actinomycin D does not inhibit protein synthesis at the concentrations studied.

Discussion

According to the current concept regarding the ACTH control of steroidogenesis, ACTH binds to the adrenal receptor (19) stimulating the adenylate cyclase enzyme which in turn triggers the synthesis of cAMP (20). cAMP then binds to the holoenzyme, protein kinase, resulting in the dissociation of the catalytic subunit (21), which through a series of events controls the transformation of cholesterol to pregnenolone (22). The precise steroidogenic step at which the translational control is exhibited by ACTH or cAMP is the transformation of cholesterol to (20S)-20-hydroxycholesterol (11,23,24).

In confirmation of the previous observations (2), the present studies show that cGMP, in addition to ACTH and cAMP, stimulates the synthesis of corticosterone in the isolated adrenal cell. Since the latter nucleotide does not have any effect on the transformation of (20S)-20-hydroxycholesterol and pregnenolone to corticosterone, it would suggest that the step stimulated by cGMP is the transformation of cholesterol to (20S)-20-hydroxycholesterol. Actinomycin D inhibits cGMP-activated steroidogenesis from endogenous precursor, presumably cholesterol, but has no effect on the incorporation of pregnenolone into corticosterone. This would imply that the step at chich transcriptional control of cGMP is manifested consists of the transformation of cholesterol to (20S)-20-hydroxycholesterol. Since the complete inhibition of cGMP-stimulated steroidogenesis is not observed with actinomycin, the possibility of both transcriptional and translational control by this nucleotide exists.

Previous (11) and present studies show that the biosynthetic step, cholesterol to (20S)-20-hydroxycholesterol is also under the translational control of cAMP, the nucleotide which presumably mediates hormonal control of ACTH. This would confirm the previous observations (13,14) that the mode of ACTH action is regulated by

both nucleotides, cGMP and cAMP. Since it takes about sixty minutes before the inhibitory action of actinomycin D is manifested on cGMP-stimulated steroidogenesis, it may be inferred that the action of cGMP, presumably in the synthesis or processing of mRNA, is quite rapid

In view of these findings, any attempt to explain the role of cGMP in the mechanism of ACTH action can only be speculative. One possibility which would account for ACTH action in stimulating steroidogenesis would be that at low concentrations the hormone stimulates guanylate cyclase activity which in turn stimulates the synthesis of cGMP (13,14). The latter nucleotide, therefore, would be responsible for the formation or processing of specific mRNA. ACTH, however, at high concentrations stimulates only the adenylate cyclase system (13); the latter stimulated system would be responsible for the synthesis of cAMP which in turn, through cAMP-dependent phosphorylation, may cause translation of the cGMP-induced mRNA. This could be one of the reasons that ACTH at physiologic concentrations does not raise the levels of cAMP (12,25,26). That ACTH stimulates the synthesis of a long lived mRNA which in turn codes for a protein essential for the cholesterol to pregnenolone conversion, has been proposed from one laboratory (27).

Unresolved, however, is the manner in which exogenous cGMP stimulates the synthesis of corticosterone without the addition of either ACTH or cAMP. This might be due to either the cross reactivity of cGMP with the cAMP-dependent protein kinase for cGMP or to the presence of a cGMP-dependent protein kinase which has a very low K_m for this nucleotide. In the latter case the involvement of another preformed mRNA which is translated by cGMP-mediated factor (s) would be necessary.

This investigation further demonstrates the advantage of the isolated adrenal cell system to study the mechanism of ACTH action

in the control of steroid hormones. The isolated adrenal cells do not have any detectable phosphodiesterase activity (28) and, therefore, the effect of the hormone can be investigated without interference from this enzyme.

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