3′,5′-Cyclic AMP and the Adrenal Desmolase System* (34084)

MARGO P. COHEN[1] AND KANAME MORIWAKI
(Introduced by Piero P. Foa)
Division of Research, Sinai Hospital of Detroit, Detroit, Michigan 48235

There is considerable evidence that 3′, 5′-cyclic AMP acts as an intracellular mediator for the adrenocorticotropic action of ACTH (1–5). For example, it has been demonstrated that cyclic AMP can simulate the effect of ACTH on the adrenals and that stimulation by ACTH produces an increase in cyclic AMP in adrenal tissue (5–7). It is of considerable interest, therefore, to determine how cyclic AMP exerts this effect. The hydroxylation of cholesterol to 20-hydroxycholesterol and that of hydroxycholesterol to 20α, 22-dihydroxycholesterol are the first steps in the biosynthesis of pregnenolone (8–10) and the rate-limiting steps in corticosteroidogenesis (11). The overall conversion of cholesterol to pregnenolone is accomplished by the cholesterol side-chain desmolase complex of the adrenal mitochondria, which includes the hydroxygenases and cleaving enzymes, and requires TPNH and molecular oxygen (12, 13). ACTH influences one or both of these hydroxylation steps (14). According to Haynes and Berthet (1), cyclic AMP would mediate the effect of ACTH by activating the phosphorylase system, ultimately resulting in the production of the required TPNH via the hexose monophosphate shunt. However, the results of recent investigations have suggested other roles for cyclic AMP in the adrenal cortex. Notably, the work of Roberts *et al.* (4, 15) and Creange *et al.* (3) has suggested that cyclic AMP stimulates pregnenolone biosynthesis by activating rate-limiting mitochondrial enzymes. However, Koritz *et al.* (16) have reported that the accumulation of pregnenolone which occurs in the presence of cyclic AMP is not due to increased biosynthesis, but rather to an inhibition of its conversion to progesterone. Recently, Satoh *et al.* (13) have stated that, in the presence of TPNH, cyclic AMP has little or no effect on the adrenal desmolase system, but no data are reported in their paper. The present study was undertaken to investigate what effect, if any, cyclic AMP has on the adrenal desmolase system and on steroidogenesis in the presence of aminoglutethimide, a potent inhibitor of cholesterol 20-hydroxylation (17).

Material and Methods. Assays for the cholesterol side chain–cleaving desmolase system were performed according to the method of Kimura *et al.* (18). In this method, an acetone powder extract of homogenized adrenal tissue is reacted with radioactive substrate in the presence of a TPNH-generating system. After incubation, the samples were heated to dryness at 135° to remove the volatile labeled side chain cleaved from the substrate. Enzyme activity was measured by the decrease in radioactivity resulting from the cleavage of labeled side chain. Details of this procedure have been described previously (20). Cholesterol-26-^{14}C (New England Nuclear) or side chain labeled 20-hydroxycholesterol-^{3}H, suspended with the aid of Tween 80 (100 μg/ml) were used as substrate. 20-Hydroxycholesterol was synthesized using the method of Petrow and Stuart-Webb (19) by a previously described procedure (17). When present, aminoglutethimide (1 μg/ml) was suspended with Tween 80 and substrate and dried. Acetone-powder extracts of fresh-frozen beef adrenal, solubilized in phosphate buffer pH 7.4, were used as the enzyme source.

Tissue incubations were performed as fol-

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[1] Trainee, Diabetes Training Grant AM 5474, National Institutes of Health. Present address: Instituto de Fisiologia, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.

lows: guinea pig adrenals were obtained immediately after sacrifice, trimmed, quartered, weighed in paired sets, and preincubated for 1 hr. About 50 mg of tissue per sample were then transferred to fresh medium and homogenized in a total volume of 1 ml. All incubations were carried out in Krebs-Ringer bicarbonate buffer containing glucose at a concentration of 200 mg/100 ml, at 37°, in an atmosphere of 95% O_2 and 5% CO_2, for 2 hr. Cholesterol-4-^{14}C (1 μCi) and, when indicated, aminoglutethimide (2 μg/ml), dissolved in benzene, were placed in the incubation beakers beforehand and evaporated to dryness.

Although this concentration of aminoglutethimide was twice that employed in the desmolase reactions, it was considerably less than the concentration previously reported to produce total blockade of steroidogenis in tissue slices (21). Cyclic AMP was added at a 2 m*M* concentration. After incubation, the tissue homogenates were extracted with dichloromethane for 1 hr and again for 30 min. The extracts were evaporated to dryness and dehydrated over sodium sulfate. The dried extracts were spotted with methanol on thin-layer chromatography plates and run in chloroform–ethanol 90:10. Ten micrograms each of pure unlabeled progesterone, corticosterone, and cortisol were added as carriers and, in addition, were spotted separately as markers. After chromatography, the fractions were detected with UV light and eluted with ethanol. The corticosterone and progesterone fractions were dried, redissolved in methanol, and rechromatographed in benzene–ethanol 90:10 after adding the carrier. The appropriate spots were eluted with ethanol and the concentration of all fractions was determined at the wave length of peak absorbance against appropriate standards, using a Gilford spectrophotometer. The samples were then dried and their radioactivity measured by liquid scintillation. Control spots of unlabeled marker were eluted with each chromatography, concentrations determined spectrophotometrically, and corrections were applied to the tissue steroid concentrations before calculation of specific activity.

Results. Table I shows that 3′,5′-cyclic AMP did not enchance the overall rate of conversion of cholesterol to pregnenolone. Data from a similar series of experiments, using 20-OH-cholesterol-^{3}H (5×10^{-9} moles) and varying concentrations of enzyme, are shown in Table II. There was no difference in the rate of substrate disappearance in the presence or absence of cyclic AMP. Since aminoglutethimide has been shown to be a competitive inhibitor of the 20-hydroxylation of cholesterol in adrenal tissue extracts, a possible effect of cyclic AMP subsequent to the 20-hydroxylation step was sought by adding both aminoglutethimide and cyclic AMP to the reaction mixture. As seen in Table III, no such effect was demonstrated. With the limited amount of substrate present in these reactions, a stimulatory effect of cyclic AMP could have been detected using suboptimal amounts of inhibitor. It should be noted that the concentration of aminoglutethimide employed was near the K_i for this system (17). These results were substantiated by the demonstration that, although cyclic AMP produced approximately a threefold stimulation of the overall synthesis of corticoids in adrenal tissue homogenates, the inhibition of steroidogenesis produced by aminoglutethimide was unaffected

TABLE I. Effect of 3′,5′-Cyclic AMP on Cholesterol Disappearance Rate.[a]

Incubation time (min):	Counts/min[b]			Rate (10^{-9} moles/hr)
	0	30	60	
Control	2462	1415	983	.72
Cyclic AMP	2398	1517	1093	.65

[a] Conditions: Cholesterol-26-^{14}C, 1×10^{-9} moles; cyclic AMP, 2 m*M*; enzyme protein 2 mg.

[b] The decrease in counts per minute during incubation is a measure of enzyme activity (See Materials and Methods).

TABLE II. Effect of 3′,5′-Cyclic AMP on 20-Hydroxycholesterol Disappearance Rate.[a]

Addition	Enzyme protein (mg)	Minutes of incubation (cpm)[b] 0	60	Rate (10^{-9} moles/hr)
None	.250	2760	2320	.8
	.750	2180	1202	2.2
	1.25	2072	1006	2.6
	2.00	2622	964	3.2
Cyclic AMP	.250	2720	2094	1.0
	.750	2052	1242	2.0
	1.25	2682	1280	2.6
	2.00	2400	900	3.15

[a] Conditions: 20-OH-cholesterol-^{3}H, 5×10^{-9} moles; cyclic-AMP, 2 mM; enzyme protein, as indicated.

[b] The decrease in counts per minute during incubation is a measure of enzyme activity (See Materials and Methods).

by the nucleotide (Table IV).

Discussion. ACTH induces an accumulation of cyclic AMP in adrenal tissue which precedes and parallels the effect on steroidogenesis produced by the tropic hormone (5). The stimulation of corticosteroidogenesis by cyclic AMP in tissue homogenates has been demonstrated to occur at a site between cholesterol and pregnenolone (3, 22), although other sites may also be affected (16, 25). ACTH influences this early pathway primarily through its effect on the 20-hydroxylation of cholesterol (14, 22, 24, 27, 28) and possibly also on the synthesis of cholesterol from acetate (25). By inference, if 3′,5′-cyclic AMP were an intracellular mediator of AC TH, one would expect it to exert an action on the 20-hydroxylation step.

The data presented in this paper show that cyclic AMP did not enhance the overall enzymatic conversion of cholesterol to pregnenolone in the presence of optimum amounts of TPNH (13) and of a TPNH-generating system (glucose 6-phosphate/glucose 6-phosphate dehydrogenase) and that the nucleotide had no specific stimulatory effect on the conversion of 20-hydroxycholesterol to pregnenolone. At least three interpretations may be offered for these findings. First, that cyclic AMP exerts its stimulatory effect by increasing the supply of TPNH only. Second, that the loss of mitochondrial integrity which occurs during the preparation of the soluble enzyme may destroy the site of action of cyclic AMP. Third, that since 20-hydroxycholesterol is a better substrate for desmolase than cholesterol, once this intermediate is formed it is converted so rapidly to pregnenolone that the overall rate of labeled cholesterol disappearance would appear equal even if the initial slow step had been stimulated. Our data support the first hypothesis.

TABLE III. Inhibition of Adrenal Desmolase Activity by Aminoglutethimide in the Presence or Absence of 3′,5′-Cyclic AMP.[a]

Addition	Minutes of incubation (cpm)[b] 0	30	60	Rate (10^{-9} moles/hr)
None	2976	1487	929	.67
Aminoglutethimide	3206	2960	2579	.18
Aminoglutethimide + cyclic AMP	3186	2984	2601	.13

[a] Conditions: Cholesterol-26-^{14}C, 1×10^{-9} moles; aminoglutethimide, 4 μM; cyclic AMP, 2 mM; enzyme protein, 2 mg.

[b] Decreased substrate disappearance reflects inhibition of enzyme by aminoglutethimide.

TABLE IV. Effect of Aminoglutethimide and Cyclic AMP on Steroidogenesis in Guinea Pig Adrenal Homogenates.

Addition	Specific activity (cpm/μmole)		
	Progesterone	Corticosterone	Cortisol
None	4212	817	2062
Cyclic AMP	14,939	2397	6416
Aminoglutethimide[a]	1738	367	106
Aminoglutethimide plus cyclic AMP[b]	2380	290	417

Incubations performed at 37° under 95% O_2 and 5% CO_2 for 2 hr in 200 mg/100 ml Kreb's-Ringer buffer with 1 μCi cholesterol-4-^{14}C.

[a] Aminoglutethimide 2 μg/ml.

[b] Cyclic AMP 2 m*M*.

However, considering the limitations of the assay procedure, the small amounts of ^{14}C-labeled cholesterol used in these experiments and the mitochondrial disturbance, if not actual disruption, which occurs during homogenization, the second and third possibilities cannot be excluded.

Cyclic AMP failed to stimulate steroidogenesis when 20-hydroxylation was blocked with aminoglutethimide. Coupled with the data from the desmolase assay experiments, these results suggest that cyclic AMP does not influence a site distal to 20-hydroxylation in the cholesterol to pregnenolone pathway. The possibility that it may affect a reaction subsequent to the formation of pregnenolone was not investigated in this study.

Summary. The effect of 3′,5′-cyclic AMP on the adrenal desmolase system and on steroidogenesis was studied in the presence or absence of aminoglutethimide. No stimulatory effect was demonstrated. The results are discussed in relation to the possible mechanism of action of cyclic AMP.

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