ACTH STIMULATION OF ADENYL CYCLASE IN ADRENAL HOMOGENATES

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Adrenocorticotropin (ACTH) as well as several other hormones elevate the intracellular concentrations of cyclic 3',5'-adenosine monophosphate (cyclic 3',5'-AMP) in hormone sensitive tissues (Sutherland, Øye, and Butcher, 1965). Apparently cyclic 3',5'-AMP is the intracellular mediator of ACTH-induced steroidogenesis in the adrenal since (a) the elevation of cyclic 3',5'-AMP concentration occurs within 1 minute after the addition of ACTH before a rise in steroid production is detected (Grahame-Smith, Butcher, Ney, and Sutherland, 1967) and (b) cyclic 3',5'-AMP, added to slices or homogenates, stimulates the synthesis of steroids (Haynes, Koritz, and Peron, 1959; Roberts, Creange and Young, 1965). We found that ACTH, added to adrenal homongenates, stimulated the conversion of ATP to cyclic 3',5'-AMP without altering the rate of cyclic 3',5'-AMP breakdown, indicating that ACTH elevates the cyclic 3',5'-AMP concentration by stimulating adenyl cyclase activity. A similar effect of ACTH on adenyl cyclase activity in ghosts and subcellular particles of fat cells has been recently reported (Rodbell, 1967).

Materials and Methods--Porcine ACTH (140 U/mg) was purchased from Sigma. Crystalline beef-pork glucagon and crystalline porcine insulin (25 U/mg) were gifts of Eli Lilly. Bovine thyrotropin (4 I.U./mg) was

a gift of Dr. Peter Condliffe, National Institutes of Health. Crystalline adenosine 5'-triphosphate disodium salt (ATP) was purchased from P-L Biochemicals, $AT^{32}P$, α-labelled at 550mC/mMole, from International Chemical and Nuclear Corporation, ^{3}H-cyclic 3',5'-AMP, 1C/mMole, from Schwarz Bioresearch, cyclic 3',5'-AMP and Dowex 50W-X8, 100-200 mesh from Calbiochem, crystalline bovine serum albumin from Armour, and theophylline from Mann.

Mouse adrenal tumor cells derived from a single clone were grown in tissue culture. This strain of cells, which responds to ACTH with an increase in steroid production, was a gift of Dr. Gordon Sato, Brandeis University (Stollar, Buonassisi, Sato, 1964; Yasumura, Buonassisi, and Sato, 1966). Adenyl cyclase was assayed by a recently developed method (Krishna, Weiss, and Brodie 1967; Weiss, and Costa, 1967; Rodbell, 1967). One to two weeks after plating, the tumor cells were scraped from the petri dish with a rubber stopper. The cells from 4 plates, 6 cm diameter, were homogenized at 1°C in about 0.6 ml of 0.25M sucrose in a Dounce homogenizer, and the protein concentration was measured by the method of Lowry et al (Lowry, Rosebrough, Farr, and Randall, 1951). ACTH was added after homogenization. Twenty-five microliters of homogenate were transferred to a 10 x 75mm test tube containing $MgCl_2$, theophylline, ATP, $AT^{32}P$, Tris-HCl buffer (pH 7.5) and albumin. The total volume of the mixture was 60 microliters, and the concentration of each component is listed in the legend to figure 1. After the addition of homogenate, the mixture was shaken at 37°C for 5-15 minutes. Then ATP (4 μmoles), cyclic 3',5'-AMP (1.25 μmoles), and ^{3}H-cyclic 3',5'-AMP (0.15 microcurie) were added; the volume of addition was 0.1 ml. This mixture was boiled for three minutes. The ^{3}H-cyclic 3',5'-AMP served to determine the recovery of cyclic 3',5'-AMP in the succeeding steps. Recoveries were 28-35%. After the addition of 0.4 ml distilled water, the contents of each tube was applied

to a Dowex-50 column (0.5 x 2cm) in the hydrogen form and the effluent discarded. The column was then washed with water, and the first 2.5 ml of effluent, containing ATP, ADP and inorganic phosphate, were discarded. The next 3.0 ml contained the cyclic 3',5'-AMP. Trace quantities of radioactive ATP, ADP, and phosphate were precipitated with $BaSO_4$ (0.17M $ZnSO_4$, 0.2 ml., followed by 0.15M $Ba(OH)_2$, 0.2 ml); after centrifugation the precipitation step was repeated. Three ml of the supernate were added to 17 ml of Bray's solution and the radioactivity measured in

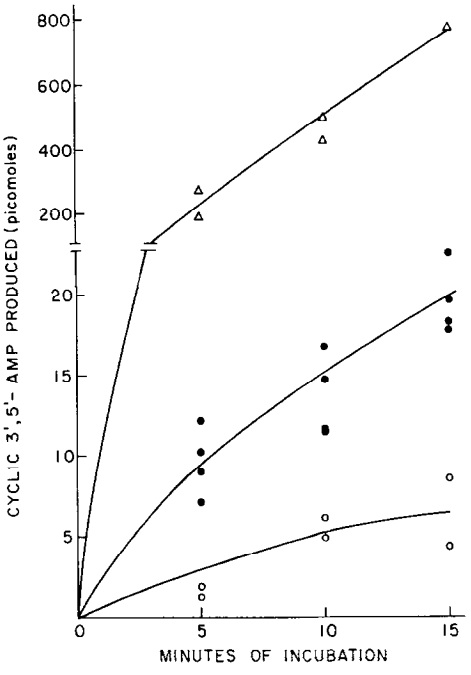

Figure 1. The Effect of ACTH and Fluoride on Cyclic 3',5'-AMP Production as a Function of Duration of Incubation. Each 0.06 ml reaction mixture contained α-labelled $AT^{32}P$, 2-3 X 10^6 cpm, ATP 1.3 mM, theophylline 8mM, $MgCl_2$ 2mM, Tris-HCl 21mM, albumin 50μg and homogenate protein 127μg. The pH was 7.5. When present, the ACTH concentration was 4.2μg/ml and sodium fluoride concentration was 9mM. ACTH was added to the homogenate at 1°, then the homogenate was added to the other components at 23°, and the reaction mixture immediately brought to 37°.

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a liquid scintillation spectrometer. ^{32}P and ^{3}H were measured simultaneously in separate channels.

Results and Discussion--ACTH added to homogenates of adrenal tumors increased the conversion of $AT^{32}P$ to cyclic 3',5'-$AM^{32}P$ (Table 1, Fig. 1). This effect was detected with homogenate protein varying from 0.37 - 1.8 mg/ml. The increase in cyclic 3',5'-AMP production due to ACTH was detected as early as five minutes after the initiation of the incubation and continued to increase for 15 minutes (Fig. 1). The effect of ACTH on cyclic 3',5'-AMP production was also observed in homogenates of normal rat adrenals (Table 2). Other peptide hormones (insulin, glucagon, thyrotropin) were without effect (Table 3).

TABLE 1

Homogenate Protein (μg)	Cyclic 3',5'-AMP Produced (Picomoles/15 minutes)		
	Control	ACTH	Fluoride
22	0.3	2.3	114
44	0.4	5.5	--
66	1.3	6.4	--
109	11.8	17.3	670

ACTH Effect on Adenyl Cyclase Activity in Adrenal Tumor Homogenates. Conditions are the same as in Figure 1. Control and fluoride represent the mean of 2 samples and ACTH the mean of 4 samples.

TABLE 2

	Cyclic 3',5'-AMP Produced (Picomoles/5 minutes)	
	Experiment 1	Experiment 2
Control	4	8
ACTH	20	36

ACTH Effect on Adenyl Cyclase Activity in Rat Adrenal Homogenates. Conditions were identical to those in figure 1 except that the homogenate in the reaction mixture was equivalent to 2 mg of adrenal (wet weight).

TABLE 3

Hormone Added	Conc. (μg/ml)	Cyclic 3',5'-AMP Produced (Picomoles/15 minutes)
None	--	10 ± 2
ACTH	4.2	21 ± 3
Insulin	4.2	11 ± 2
Glucagon	4.2	9 ± 2
TSH	12.6	10 ± 3

Specificity of the ACTH Effect on Adenyl Cyclase Activity in Adrenal Tumor Homogenates. Conditions were identical to those in the legend to figure 1. Each value is the mean of 4 samples ± SE of mean. The results of two separate experiments were combined.

The increase in cyclic 3',5'-AMP concentration produced by ACTH could have been due to either increased production or decreased destruction. The latter was excluded since under the conditions of the adenyl cyclase assay ACTH was without effect on the rate of disappearance of cyclic 3',5'-AMP (Table 4).

Fluoride increased cyclic 3',5'-AMP production, as was shown previously in beef adrenal homogenates (Haynes, 1958). When $MgCl_2$ was omitted, cyclic 3',5'-AMP production was slight or absent, even in presence of fluoride. When theophylline was omitted, cyclic 3',5'-AMP production was very markedly reduced, presumably due to hydrolysis of nascent nucleotide by the cyclic 3',5'-nucleotide phosphodiesterase. Boiled homogenates were inactive.

Since adenyl cyclase occurs in the plasma membrane of the fat cell, liver cell, and erythrocyte (Rodbell, 1967; Sutherland, Rall, and Menon, 1962; Davoren and Sutherland, 1963), it is likely that adenyl cyclase

TABLE 4

Minutes of Incubation	Cyclic 3',5'-AMP Hydrolyzed (Picomoles)	
	Control	ACTH
5	28 30	29 34
15	76 76	79 75

Failure of ACTH to Alter the Rate of Cyclic 3',5'-AMP Hydrolysis in Adrenal Tumor Homogenates. Incubation conditions were identical to those in Fig. 1 except that ATP was omitted, each reaction mixture contained 667 picomoles of ^{3}H cyclic 3',5'-AMP (116,000 c.p.m.) and 120 mg of homogenate protein. Individual values from a typical experiment are shown.

in the adrenal is associated with the plasma membrane. Recent studies showed that the first step in ACTH action, both in fat cells and adrenals, is rapid equilibration between hormone in the medium and sites on the plasma membrane, i.e. ACTH + SITE $\rightleftarrows$ ACTH-SITE (Taunton, Roth, and Pastan, 1967). Though adenyl cyclase may be the actual ACTH binding site, it seems more likely that ACTH binding occurs one or more steps before activation of adenyl cyclase. Nevertheless, because ACTH activates adenyl cyclase in broken cells, we suspect that the ACTH binding site and adenyl cyclase are anatomically quite close.

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