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The Stimulation by the Adrenocorticotropic Hormone of the Phosphorylation of an Adrenal Mitochondrial Preparation (40167)

GIRIJA BHARGAVA, ELAINE SCHWARTZ, AND SEYMOUR B. KORITZ

Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029

A possible explanation for the acute effect of the adrenocorticotropic hormone (ACTH) on adrenal steroidogenesis involves an increase in the synthesis of pertinent protein(s). In the initial test of this possibility amino acid analogues were found to inhibit protein synthesis 20–30% without affecting the steroidogenic response to ACTH (1). Ferguson, using puromycin, a more potent inhibitor of protein synthesis, found that it abolished the steroidogenic response to both ACTH and 3′,5′ cyclic AMP (cAMP) (2) and Garren *et al.* found cycloheximide to have the same effect and presented evidence that a protein(s) with a rapid turnover rate ($T_{1/2}$ of about 8 min) was involved (3). In addition, the inhibitors of protein synthesis affected steroidogenesis at the mitochondrial transformation of cholesterol to pregnenolone, the same site where ACTH and cAMP stimulate steroidogenesis (4). However, a number of investigators have reported that both puromycin and cycloheximide, under proper experimental conditions, inhibited protein synthesis with little effect on the stimulation of steroidogenesis by ACTH or cAMP (5–7). In a study of this apparent contradiction it was found that at low concentrations of puromycin or cycloheximide a much greater inhibition of protein synthesis than of cAMP-stimulated steroidogenesis occurred (7). At these low concentrations varying degrees of recovery of the rate of steroidogenesis take place with increasing time of incubation while the inhibition of protein synthesis continues in a linear manner. The recovery can be eliminated if the concentration of cAMP is reduced. These data have suggested that the concentration of the rapidly-turningover protein (RT-protein) is independent of the level of cAMP. However, the concentration of cAMP determines the extent of transformation of the RT-protein to a form active in the stimulation of steroidogenesis.

Since cAMP is known to activate protein kinases, a reasonable first assumption is that the activation of the RT-protein consists of a phosphorylation. Using gel electrophoresis we have detected a phosphorylated peak in adrenal mitochondria. This peak is increased if the tissue was exposed to ACTH and the ACTH effect sharply decreased if puromycin or cycloheximide was also present.

Methods. Beef adrenals were obtained from a local slaughter house. After the fat was removed, they were cut into sections which were placed in cold 0.154 *M* NaCl and the sections sliced with a Stadie-Riggs tissue slicer. After slicing off the capsule the first slice of the cortex was discarded and subsequent slices of the cortex placed in cold Krebs-Ringer bicarbonate solution containing 200 mg glucose per 100 ml (KRB-glucose). The slices (250–300 mg) were preincubated for 40 min in 3.0 ml of KRB-glucose under 95% O_2:5% CO_2 at 37°. After the preincubation the slices were removed, blotted on filter paper, and placed in fresh medium consisting of 2.75 ml of KRB-glucose and where indicated an excess of ACTH (3 U), 1.7 m*M* puromycin or 0.2 m*M* cycloheximide. The volume was made up to 3.0 ml with 0.154 *M* NaCl. This final incubation was carried out for 30 min at 37° under 95% O_2:5% CO_2. At the end of the incubation samples of the medium were taken for corticosteroid assay (8) and the results expressed in terms of corticosterone. The tissue slices, after blotting on filter paper were homogenized in 0.25 *M* sucrose-12 m*M* Tris, pH 7.5, to make a 10% homogenate. The mitochondria were isolated as described elsewhere (8), were not washed and were taken up in a volume of sucrose-Tris to yield a concentration of about 6–8 mg protein per ml as determined by the method of Lowry *et al.* (9).

The medium for the phosphorylation reaction contained, at final concentrations, 50

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m*M* sodium acetate–90 m*M* Tris-Cl (pH 6.4), 4 m*M* theophylline, 2.5 m*M* $MgCl_2$, 0.2% Triton X-100, 0.01 ml mitochondria, 5 μl of [γ-^{32}P]ATP (25 Ci/nmole) containing 1.5–2 × 10^6 cpm and water to a final volume of 0.10 ml. All additions were made except for the [γ-^{32}P]ATP and the reaction mixture permitted to stand at 20° for 2 min before the reaction was started with the [γ-^{32}P]ATP. Incubations were carried out at 20° for the indicated times and the reaction was terminated by the addition of 50 μl of the "SDS-stop solution" as described by Maeno *et al.* (10). The reaction mixture was then incubated at 37° for 5 min and a 100 μl aliquot subjected to polyacrylamide gel electrophoresis according to the method of Weber and Osborn (11). The electrophoresis was carried out at 2 mA per gel and usually for about 15 hr. The gels were cut into 1 mm sections and the sections, after treatment with Protosol at 55° overnight, were counted in 0.7% Omnifluor in toluene in a scintilation counter. ATP was determined by the glucose-6-phosphate dehydrogenase method (12).

Results. Mitochondria were isolated from adrenal cortex slices which had been incubated with and without ACTH and with ACTH plus puromycin and incubated with [γ-^{32}P]ATP in the standard phosphorylation reaction mixture. The distribution of radioactivity following gel electrophoresis in such an experiment is given in Fig. 1. The increase in phosphorylation caused by ACTH is apparent as is the effect of puromycin on the ACTH effect. The results of two other experiments of this type are given in Table I. It is apparent in all the experiments that ACTH treatment of the tissue slices increases the phosphorylation of the corresponding mitochondria and that inhibitors of protein synthesis decrease the ACTH effect, at times to values below the control value. It is also clear that the changes in the extent of phosphorylation are accompanied by corresponding changes in corticoid synthesis. The effect of ACTH on the mitochondrial phosphorylation is usually most apparent at short incubation times such as 30 sec. In one experiment the ACTH effect was +62% at 15 sec and no effect was seen at 1 min. In another experiment the ACTH effect was +170% at 1 min and +15% at 2 min. This appears to be due primarily to the loss of phosphate from the ACTH peak, suggesting the presence of phosphatase activity. It is perhaps this dephosphorylation combined with the fixed amount of ^{32}P-ATP present which accounts for the relatively limited existence of the phosphorylated material present in the experimental sys-

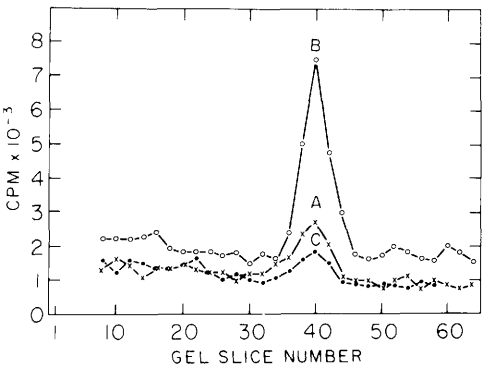

FIG. 1. The effect of the incubation of beef adrenal cortex slices with ACTH and ACTH plus puromycin on the phosphorylation by (γ-^{32}P)ATP of the isolated mitochondria as shown by gel electrophoresis. Curve A, control; curve B, ACTH; curve C, ACTH plus puromycin. Steroid synthesis, nmoles per g tissue, in the control, ACTH and ACTH plus puromycin incubations was 7.7, 13.2 and 5.3 respectively and the mitochondrial protein present in the corresponding phosphorylation reactions was 86 μg, 82 μg and 78 μg and the incubation time was 1 min.

TABLE I. THE PHOSPHORYLATION OF MITOCHONDRIA ISOLATED FROM ADRENAL CORTEX SLICES SUBJECTED TO VARIOUS TREATMENTS.[a]

Treatment of tissue slices	Phosphorylation cpm/ng protein	Corticoid synthesis nmoles/g tissue
Experiment 1		
Control	9.3 ± 0.8	23.3 ± 0.3
ACTH	37.9 ± 1.2	55.1 ± 0.4
ACTH + Puromycin	15.2 ± 0.7	21.9 ± 0.4
ACTH + Cycloheximide	20.5 ± 0.9	19.1 ± 0.3
Experiment 2		
Control	38.2	14.1 ± 0.3
ACTH	62.0	28.3 ± 0.4
ACTH + Puromycin	33.4	14.2 ± 0.4

[a] The incubation time was 45 sec. Phosphorylation refers to the total cpm in the peak (Fig. 1) calculated to a phosphorylation mixture volume of 0.1 ml.

tem in its current state.

Substituting the cytosol or the microsomes for the mitochondria in the phosphorylation reaction mixture resulted in no peak of radioactivity on the polyacrylimide gel. In addition, the mitochondrial phosphorylation appears to be specific for the adrenal cortex since no peak of radioactivity could be detected with tissue from the medulla in an experiment where 510 cpm were found with cortical tissue.

Since changes in mitochondrial ATP levels would affect the specific activity of the ^{32}P-ATP in the phosphorylation reaction mixture, these levels in the mitochondria used in experiment 2, Table I, were determined. These were found to be 71, 91, and 72 nmoles ATP per ml for the control, ACTH, and ACTH plus puromycin mitochondrial suspensions used. It is clear that this factor cannot account for the observations.

The data in Table II show that the presence of the detergent, Triton X-100, is essential for the phosphorylation reaction to take place. Disruption of the mitochondria by treatment with a hypotonic medium or by two cycles of freezing, in the presence or absence of Ca^{2+}, were without effect (Table III). It has been reported in several cases that protein kinase activity associated with cellular membranes becomes observable only in the presence of Triton X-100 (13, 14).

Discussion. The mitochondrial component(s) which is phosphorylated is not known. The position of migration on the polyacrylamide gel indicates a substance, or an association with a substance, of high molecular weight. Initial estimates using the method of Weber and Osborn (11) indicate a molecular weight of 42×10^3 to 45×10^3 daltons. We also have no direct evidence that the phosphorylated component is involved in cholesterol side-chain splitting activity since Triton X-100 inhibits this activity. It does appear, however, that the phosphorylated component is related to the action of ACTH on steroidogenesis. The hormone stimulates the transformation of cholesterol to pregnenolone, which occurs in the mitochondria (15). The stimulation involves a rapidly turning over protein (3), the concentration of which appears to be independent of ACTH (7). On this basis it has been suggested that ACTH affects the transformation of this protein to a form which stimulates steroidogenesis. This possibility is strengthened by the report of Caron *et al.* (16) that the activity of a reconstituted cholesterol side-chain cleavage system is increased by the addition of ATP and a protein kinase. We have found that isolated adrenocortical mitochondria can be phosphorylated and the extent of phosphorylation is increased by exposure of the tissue to ACTH prior to the isolation of the mitochondria. This effect of ACTH is severely reduced, or abolished, if inhibitors of protein synthesis are present along with the ACTH. Since only a 30 min incubation is used, these results indicate that a protein of short half-life is involved. In addition, the extent of phosphorylation and the synthesis of corticosteroids in the tissue slices change in the same directions.

TABLE II. THE EFFECT OF TRITON X-100 ON THE PHOSPHORYLATION OF MITOCHONDRIA.[a]

μl 2% Triton X-100	Phosphorylation cpm
0.3	125
3.0	870
10.0	1640

[a] The final volume of the phosphorylation incubation mixture was 0.10 ml and the incubation time was 1 min. The mitochondria used were isolated from tissue slices treated with ACTH.

TABLE III. THE EFFECT OF VARIOUS TREATMENTS OF THE MITOCHONDRIA ON THE EXTENT OF PHOSPHORYLATION.[a]

Treatment of mitochondria	Phosphorylation cpm
Control	1260
Water	<150
Water + Ca^{2+}	<150
Water, freeze-thaw 2×	<150
Water, freeze-thaw 2× + Ca^{2+}	<150

[a] The control incubation contained all the components of the standard phosphorylation reaction mixture. Other conditions are as in the legend to Table II. Water treatment refers to mitochondria which had been suspended in water and the Triton X-100 omitted from the phosphorylation reaction mixture. Ca^{2+}, when present, was added to the phosphorylation reaction mixture at a final concentration of 11 m*M*.

Summary. Mitochondria isolated from beef adrenal cortex slices which had been incubated with and without ACTH and with ACTH plus puromycin or cycloheximide were incubated with (γ-^{32}P)ATP in the pres-

ence of the detergent, Triton X-100. A phosphorylated peak, detected by gel electrophoresis, was increased by ACTH and sharply decreased if the inhibitors of protein synthesis were present along with the ACTH. The changes in the extent of phosphorylation are accompanied by corresponding changes in corticoid synthesis. The data suggests that ACTH causes the phosphorylation of a rapidly turning-over protein which stimulates the conversion of cholesterol to pregnenolone.

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