The marked dissociation between cyclic AMP and corticosterone production during stimulation of adrenal cells with adrenocorticotropic hormone (ACTH) has questioned the intermediate role of cyclic AMP in steroidogenic responses to low concentrations of ACTH. This discrepancy was resolved by assay of the putative active fraction of cyclic AMP, i.e. bound to the regulatory subunit of protein kinase, during measurement of cyclic AMP pools and corticosterone production in isolated rat adrenal cells. In collagenase-dispersed fasciculata cells, corticosterone production was stimulated by 1 pm ACTH, with maximum response at 1 nm ACTH and ED₅₀ of 100 pm ACTH. Receptor-bound, intracellular, and extracellular cAMP were increased by 1 pm ACTH and rose progressively with the serial increase in corticosterone production evoked by higher ACTH concentrations. During kinetic studies, increases in receptor-bound and intracellular cyclic AMP were found to be concomitant with hormone-induced steroid elevations. Preincubation of adrenal cells was necessary to observe the parallel stimulation of both cyclic AMP and corticosterone by ACTH over the lowest range of hormone-induced responses (from 10⁻¹² to 10⁻¹⁰ M). Receptor-bound cyclic AMP rose to twice the basal value as corticosterone increased to its maximum at 1 nm ACTH, and rose 6-fold at 100 pm ACTH. The free receptor sites for cAMP, measured by [³H]cAMP binding, decreased with the rising occupancy of receptors by endogenous cyclic AMP produced during ACTH stimulation. The demonstration that small increments in cAMP bound to receptor protein consistently accompany stimulation of corticosterone by trophic hormone provides evidence for the intermediate role of cyclic AMP in the action of ACTH on adrenal steroid secretion.

AMP pools and detailed analysis of cyclic AMP bound to the regulatory subunit of protein kinase in Leydig (6, 7) and luteal cells (8).

To evaluate the role of cyclic AMP during hormone action in adrenal fasciculata cells, we have analyzed the relationships between cyclic AMP production, binding of the nucleotide to its receptor protein, and stimulation of corticosterone synthesis in rat adrenal fasciculata cells during the action of low concentrations of ACTH.

MATERIALS AND METHODS

Male Sprague-Dawley rats (200 to 250 g) were killed by decapitation and the adrenal glands were rapidly removed and kept in Medium 199 at room temperature. After removal of adipose tissue, the capsular zone was separated from the medulla and fasciculata-reticularis layers by squeezing each adrenal firmly within a cellulose tissue. For dispersion of cells, the inner adrenal tissue was minced with scissors, and the fragments were washed with Medium 199. The washed tissue mince was then incubated at 37°C in Medium 199 containing collagenase type II (Worthington) (2 mg/ml), DNase (Sigma, type H-II) (25 μg/ml), and bovine serum albumin (2 mg/ml). After incubation for 10 min, the enzyme-treated tissue was dispersed by repeated aspiration through a 12-cm length of polyethylene tubing (4-mm inside diameter) attached to a 20-ml disposable plastic syringe, followed by decantation of the turbid supernatant (9). This procedure was repeated on the tissue particles until a clear supernatant was obtained. The pooled supernatants containing the dispersed cells were filtered through five layers of surgical gauze into 250-ml plastic bottles, which were centrifuged at 120 × g for 10 min at 4°C. The cell pellets were suspended in Medium 199 containing 0.2% bovine serum albumin and incubated batchwise for 2 h at 37°C. After this preincubation step, the cells were collected by centrifugation and suspended in Medium 199 containing 0.2% bovine serum albumin to a final concentration of about 250,000 cells/ml.

Cell Incubation and Sample Preparation—Incubations were performed in polyethylene vials (Packard) containing 1 ml of cell suspension at 37°C under 95% O₂, 5% CO₂ in the absence and presence of porcine ACTH (Sigma, 150 units/mg) added as 100-μl aliquots to the sample vials. Unless indicated, all incubations were performed in the presence of 0.125 mM 1-methyl-3-isobutylxanthine. After selected time intervals, the incubations were terminated by transferring the vials to an ice bath, and all further steps were performed at 0-4°C. The contents of each vial were transferred to 16-ml polyethylene tubes and centrifuged at 250 × g for 10 min. The supernatant solution was assayed for corticosterone and extracellular cyclic AMP released into the incubation medium. For assay of extracellular cyclic AMP, 900 μl of medium was placed in tubes containing 100 μl of 10 mM theophylline (Sigma), transferred to a boiling water bath for 12 min, and stored frozen until analyzed.

Preparation of Cell Extracts for Assay of Intracellular and Receptor-bound Cyclic AMP—This procedure was performed essentially as previously described (6). Briefly, cells were washed once with ice cold Medium 199 containing 0.1% bovine serum albumin and resuspended in 1 ml of phosphate-buffered saline, pH 7.4, containing 1 mM theophylline. After sonication of the cell suspension for 15 s, 600-μl aliquots were filtered through 0.45-μm HAWP cellulose membranes (Millipore). The filters were then placed in glass tubes containing 900 μl of 1 mM theophylline in phosphate-buffered saline, and
heated for 10 min in a boiling water bath. The cyclic AMP released from its filter-adsorbed binding protein (i.e. the regulatory subunit of protein kinase) was analyzed by radioimmunoassay. The remaining 400 µl of sonicated cell suspension was heated at 100°C for 10 min and subsequently analyzed for total intracellular cyclic AMP (i.e. free plus receptor-bound nucleotide).

Measurement of Corticosterone and Cyclic AMP—Corticosterone was measured by the radioimmunoassay procedure of Ruder et al. (10). Radioimmunoassay of cyclic AMP was performed by a modification (6, 11) of the method of Steiner et al. (12), with addition of the acetylation step described by Harper and Brooker (13).

Cyclic AMP Binding assay—Samples for cyclic AMP binding assays were obtained from incubations performed in parallel with those described above. The cell pellets were resuspended in 500 µl of 20 mM Tris/HCl buffer, pH 6.8, containing 1 mM methylisobutylxanthine. Aliquots of the extract (100 µl equivalent to 1 to 1.5 x 10⁶ cells) were incubated with 200 µl of [³H]cyclic AMP (30 Ci/mmol, New England Nuclear) in 50 mM phosphate buffer, pH 6.8, containing 20 mM theophylline, magnesium acetate, 10 mM 2-mercaptoethanol, and 100 µl of the buffer containing 1 mM methylisobutylxanthine. After incubation at 6°C for 2 to 3 h, or for specified times in individual experiments, the [³H]cyclic AMP bound to protein kinase was isolated by adsorption to cellulose filters (14). Nonspecific binding was determined in the presence of 5 µM cyclic AMP.

Calculations of Binding Data and ED₅₀ Values—The data were analyzed by an equation relating the concentration of bound ligand to the total cyclic AMP concentration and kinetic studies were analyzed with a nonlinear curve-fitting computer program (15). The ED₅₀ values for corticosterone responses to human chorionic gonadotropin and the derivation of maximal steroid production were calculated using a four-parameter logistic program (16).

RESULTS AND DISCUSSION

Initial studies on the dose-related effects of ACTH (10⁻¹³ to 10⁻⁰ M) on corticosterone production by several concentrations of adrenal cells were performed in the presence and absence of methylisobutylxanthine. Incubation of 280,000, 480,000, and 980,000 adrenal cells/ml (cell yield obtained for one, two, or four adrenal glands), gave corresponding ED₅₀ values for ACTH of 76.5 ± 5 x 10⁻¹² M and 149.3 ± 10⁻¹² M, respectively. Thus, reduced sensitivity of dispersed cells to ACTH was observed with increasing numbers of cells. In addition, dose-response curves performed with 280,000 cells in the absence of methylisobutylxanthine showed only a small shift or reduction in sensitivity to ACTH, with ED₅₀ of 102.5 ± 5 x 10⁻¹² M ACTH. All further experiments were performed with 280,000 cells/ml to provide optimal sensitivity to ACTH.

Kinetic studies on the stimulation of cyclic AMP production by ACTH were performed with freshly dispersed adrenal cells. When the time course of cyclic AMP production in response to a near-maximal steroidogenic concentration of ACTH (10⁻¹⁰ M) was examined, significant increases in intracellular and receptor-bound cyclic AMP were observed at all times from 2 to 60 min. Early (2 to 15 min) transient rises above the control values of intracellular and receptor-bound cyclic AMP were followed by a decline to values that were slightly higher than the zero time level, remaining relatively constant for incubation times up to 60 min.

The intracellular cyclic AMP responses to ACTH were increased 1.5- to 2-fold over control values, and similar increases were evident in the receptor-bound cyclic AMP. Extracellular cyclic AMP rose progressively during incubation, being significantly elevated at 5 min and reaching a maximum of 20 times the control value after 60 min. Corticosterone production was also significantly elevated after 5 min, and rapidly increased during the incubation period to 250 ng/10⁵ cells at 60 min, a 100-fold stimulation over the control values, which rose only 2-fold during incubation.

We also performed kinetic studies using a lower ACTH concentration (10⁻¹⁰ M) that was closer to the ED₅₀ for corticosterone stimulation. In this experiment, there was no apparent stimulation of bound or intracellular cyclic AMP above the control incubations. However, there was again a transient rise in basal cyclic AMP production, with an increase of 100% in both bound and intracellular cyclic AMP between 2 and 15 min, declining thereafter almost to control levels (Fig. 1). In contrast, significant increases in steroid production were observed at 5 min, with a progressive increase at later times to a maximum at 60 min, and a 20-fold increase above the control values.

These observations suggested that isolated adrenal cells initially behaved as though ACTH-stimulated, with increases in bound and intracellular cyclic AMP during the first 15 min of incubation in both control and hormone-stimulated cells. In all subsequent experiments, the dispersed cells were preincubated for 2 h before hormone stimulation to provide more constant basal conditions.

More detailed kinetic experiments were performed upon preincubated adrenal cells stimulated with a near-maximal ACTH concentration (10⁻⁹ M) and a concentration (10⁻¹¹ M) below the ED₅₀ for corticosteroid production. Both ACTH concentrations caused significant elevations of extracellular cyclic AMP at 2 and 5 min (p < 0.01) and at all times thereafter (Fig. 2, left). The higher ACTH concentration (10⁻⁹ M) evoked a maximum response of 20 pmol of cyclic AMP/10⁵ cells after a 30-min incubation, a 500-fold increase above the control value. Intracellular cyclic AMP was increased after 2 min with the higher dose, reaching the maximum level of 1.6 pmol/10⁵ cells at 30 min, a 5-fold stimulation above the control value. This was followed by a decrease of between 30 and 60 min and a slight rise again at 120 min. With the lower ACTH dose, analysis of variance and combined t-test showed a significant increase above the controls at times from 5 to 30 min, with a further increase between 60 and 120 min (Fig. 2, middle). Receptor-bound cyclic AMP levels were increased significantly after 2 min with the high ACTH dose, reaching a maximum at 15 min of 350 fmol/10⁵ cells, a 2.4-fold increase above the control level that was maintained up to 120 min.

![Fig. 1. Intracellular and receptor-bound cyclic AMP in adrenal cells stimulated with 10⁻¹⁰ M ACTH for 2 to 60 min. Cells were not preincubated prior to stimulation. Each point represents the mean ± S.E. of triplicate incubations.](http://www.jbc.org/content/12/2/3862/(35x729)
Fig. 2. Left, release of cyclic AMP into extracellular medium of adrenal cells incubated with ACTH (10^{-8} and 10^{-11} M) and from control incubations for 2 to 60 min. The cells used in these and subsequent experiments were preincubated for 2 h prior to stimulation. Each point represents the mean ± S.E. of triplicate incubations. Middle, changes in total intracellular cyclic AMP of adrenal cells stimulated with 10^{-8} and 10^{-11} M ACTH for 2 to 120 min. Cells were preincubated for 2 h prior to stimulation. Each point represents the mean ± S.E. of triplicate incubations.

Fig. 3. Release of corticosterone into extracellular medium of adrenal cells incubated with ACTH (10^{-8} and 10^{-11} M) for 2 to 60 min. Cells were preincubated prior to stimulation for 2 h. Each point represents the mean ± S.E. of triplicate incubations.

(Fig. 2, right). The lower ACTH concentration caused a small and sustained increase in receptor-bound cyclic AMP that was significantly higher (p < 0.01) than the control values. Corticosterone production was significantly increased by 10^{-8} M ACTH after 2 min, rising to 600 ng/10^5 cells at 120 min, a 12-fold increase over control (Fig. 3). The lower ACTH concentration stimulated corticosterone production after 5 min, reaching a 2-fold increase after 120 min. A comparative study of corticosterone production and cyclic AMP levels was performed over a wide range of ACTH concentrations (10^{-12} to 5 \times 10^{-8} M) in the presence of 0.1 mM methylisobutylxanthine. In each case, dose-related increases were observed in intracellular, receptor-bound, and extracellular cyclic AMP when measured over the ACTH concentration range that elicited a full corticosterone response (Fig. 4). The basal corticosterone production increased from 1.25 pg/10^5 cells to 5.4 pg/10^5 cells during incubation for 2 h. In most cases, corticosterone production was significantly increased by incubation with 10^{-12} M ACTH, with maximum steroid levels at 10^{-9} M ACTH and ED_{50} of 10^{-10} M ACTH.

Extracellular cyclic AMP was significantly increased at ACTH concentrations of 10^{-12} M (p < 0.05), 10^{-11} M (p < 0.05), and 10^{-10} M and above (p < 0.01). Bound cyclic AMP
was increased significantly over control values at 10^{-12} \text{ M ACTH} (p < 0.05) at 10^{-11} \text{ M ACTH} (p < 0.01). Maximal bound cyclic AMP values were attained at ACTH concentrations between 10^{-7} and 10^{-6} \text{ M}, with a 2-fold total increase from control values of 600 fmol/10^6 cells to 1200 fmol at the dose of ACTH (10^{-9} \text{ M}) that elicited maximal corticosterone responses, and to 2400 pmol at 10^{-7} \text{ M ACTH}. Thus, a 2-fold increase in bound cyclic AMP was observed when corticosterone reached maximum values at 10^{-10} \text{ M ACTH}, and further increases of bound cyclic AMP at the higher ACTH concentrations did not provoke further changes in corticosterone stimulation (Fig. 5).

Binding studies on the interaction of [^3H]cyclic AMP with the adrenal receptor protein showed that the affinity constant ($K_a$) for cyclic AMP binding, calculated from equilibrium data after incubations at 6°C for 17 h, was $3.0 \times 10^8 \text{ M}^{-1}$, and Scatchard plots revealed a single order of binding sites. During kinetic studies, the association and dissociation of [^3H]cyclic AMP with the binding protein were analyzed at 6°C and 0°C. The association rate constant ($k_1$) was $8.1 \pm 0.52 \times 10^4 \text{ min}^{-1}$ at 6°C, and the dissociation rate constant ($k_2$) was $9.7 \pm 0.20 \times 10^{-4} \text{ min}^{-1}$ at 6°C and $5.9 \pm 0.19 \times 10^{-4} \text{ min}^{-1}$ at 0°C. The association constant computed from the rate constants measured at 6°C ($1.37 \times 10^9 \text{ M}^{-1}$) was similar to the $K_a$ derived by Scatchard analysis of the equilibrium binding data.

The data obtained during dissociation studies indicated that it should be possible to measure free cyclic AMP receptors during ACTH stimulation, to follow changes in occupancy during hormonal stimulation. Analysis of free cyclic AMP receptors by [^3H]cyclic AMP binding assay was performed after hormonal stimulation of adrenal cells by ACTH concentrations (10^{-12} to 10^{-8} \text{ M}) that stimulated corticosterone production in a dose-related manner, and at higher ACTH concentrations that increased cyclic AMP formation while corticosterone remained constant at the maximum level. A dose-dependent decrease of free cyclic AMP receptor sites was readily apparent over the ACTH concentration range that evoked a full corticosteroid response, and a further decrease in free sites was observed with supramaximal steroidogenic doses up to 10^{-5} \text{ M ACTH} (Table I).

These studies have shown significant elevations of receptor-bound cyclic AMP over the entire ACTH dose-response range for corticosterone responses (10^{-12} to 10^{-9} \text{ M}). A parallel decrease of free cyclic AMP receptor sites was detected during binding studies with [^3H]cyclic AMP under nonexchange conditions, and kinetic studies always showed correlation between cyclic AMP bound to receptor sites and steroidogenesis. These results are consistent with an intermediate role of cyclic AMP and cyclic AMP-dependent protein kinase in the acute steroidogenic response of fasciculata cells to physiological levels of trophic hormone. Recent studies demonstrating increases of cyclic AMP-independent activity of phosphoprotein kinase in human (5) and rat adrenal cells (4) by low doses of ACTH have provided additional supportive evidence for this mechanism. The lack of detection of cyclic AMP increases in these and earlier reports (1-5) was resolved in this study by a combined approach of optimization of incubation conditions, the use of a highly sensitive assay for cyclic AMP, and direct measurement of receptor-bound cyclic AMP.

It is evident that about 25% of cyclic AMP receptors need to be occupied to produce the maximal corticosterone response elicited by 10^{-11} to 10^{-8} \text{ M ACTH}, and that further increases in cyclic AMP receptor occupancy did not evoke changes in the maximum steroid production. These findings are similar to our previous observations in Leydig cells (6, 7), and differ in degree from those in luteal cells (8) where major receptor occupancy was necessary to attain maximal progesterone responses. In all three steroidogenic tissues, changes in extracellular cyclic AMP were detectable in parallel with increasing receptor-bound nucleotide, and in the testis and adrenal were relatively greater in magnitude than those in receptor-bound cyclic AMP. While the latter gives an accurate measure of active nucleotide concentrations in hormone-stimulated cells, the extracellular accumulation of cyclic AMP (especially in the presence of a phosphodiesterase inhibitor) is a sensitive index of nucleotide production.

This demonstration of an involvement of cyclic AMP at all levels of hormone-stimulated steroidogenesis is in contrast...
with the view that cyclic GMP might act as the second messenger for ACTH action in the adrenal gland (4). In recent studies, we have found that cyclic GMP changes are poorly correlated with steroidogenesis in ACTH-stimulated adrenal cells, and that increases in cyclic GMP are largely confined to the extracellular compartment, with no detectable changes in intracellular levels. Also, it requires much higher concentrations of cyclic GMP analogues than the corresponding cyclic AMP derivative to evoke corticosterone responses in isolated adrenal cells (17). While the evidence for a role of cyclic GMP in ACTH action remains incomplete, the present findings have satisfied a further requirement of the evidence that cyclic AMP acts as mediator of the steroidogenic effects of ACTH (18). These observations, together with recent studies on the actions of gonadotropins (6-8), have adequately demonstrated that small changes in cyclic AMP production and binding to protein kinase receptors are involved in the acute regulation of steroidogenesis by trophic hormone in the adrenal, testis, and ovary.

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*J. Biol. Chem.* 1979, 254:3861-3865.

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