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Mechanism of Corticotropin and cAMP Induction of Mitochondrial Cytochrome P450 System Enzymes in Adrenal Cortex Cells*

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We studied the kinetics of corticotropin (ACTH) induction of mitochondrial cytochromes $P450_{scc}$ and $P450_{c11}$ and their electron transport proteins, adrenodoxin and adrenodoxin reductase, in bovine adrenal cortex cells in primary culture. The mRNA levels of these enzymes increase and reach a peak within 3–12 h after ACTH addition. The protein levels of adrenodoxin reductase and $P450_{scc}$ show an increase only nearly 24 h after ACTH addition. After ACTH addition, the intracellular level of cAMP reaches maximal levels within 5 min, and then decreases gradually over 60 min. Hence, we examined the effect of a pulse of ACTH or cAMP analogs on enzyme and mRNA levels. Exposure of the cells to ACTH for 1–2 h was sufficient for maximal induction of the enzymes and $P450_{scc}$ mRNA. In contrast, the induction of the enzymes and the mRNA by cAMP analogs or forskolin required the continuous presence of these agents for over 12 h. But, these agents stimulated cortisol secretion to the medium quickly, indicating that they can activate some intracellular processes while not showing any effect on enzyme induction. The absence of any effect of prolonged cAMP pulses on enzyme and mRNA levels weakens the previous hypothesis that cAMP is the sole second messenger for the ACTH induction of steroidogenic enzymes in adrenal cortex cells. The inductive ability of a brief pulse of ACTH indicates that ACTH can rapidly initiate a series of reactions that result in enzyme induction many hours later.

The first and rate-limiting step in the biosynthesis of steroid hormones is the conversion of cholesterol to pregnenolone. This reaction is catalyzed by a mitochondrial cytochrome P450 system that includes three enzymes (adrenodoxin reductase, adrenodoxin, and $P450_{scc}$) which together constitute an electron transport chain (1–3). These enzymes are present in steroidogenic cells in the adrenal cortex, ovary, testis, and placenta. In the adrenal cortex there is an additional mitochondrial P450 ($P450_{c11}$) which catalyzes both 11β- and 18-hydroxylation reactions necessary for the biosynthesis of glucocorticoids and mineralocorticoids (for reviews see Refs. 1, 4–6).

The maintenance of the normal levels of the major steroidogenic cytochromes P450 in the adrenal cortex is dependent on the stimulation of this tissue by its trophic hormone ACTH[1] (7). In adrenal cortex cells *in vitro*, the synthesis of $P450_{scc}$, $P450_{c11}$, and adrenodoxin and their mRNAs can be stimulated by ACTH and cAMP as the putative intracellular mediator of ACTH (6, 8–20). The increase in the levels of these mRNAs can be detected only after at least several hours of stimulation by ACTH or cAMP. This is quite slow relative to the fast (<1 h) induction of many genes by cAMP in different tissues (21).

Normally ACTH is secreted in pulses with a circadian pattern and the blood level of ACTH shows a peak that lasts only a few hours (22). Previous *in vitro* studies on P450 induction have examined the effects of continuous exposure of the cells to ACTH or cAMP as its putative second messenger (8–20). This type of stimulation is greatly different from the normal physiological situation. In this study we examined the kinetics of induction of all four enzymes of the mitochondrial P450 system in the adrenocortical cells in primary culture, in response to continuous as well as pulsatile exposure of the cells to ACTH and cAMP analogs.

Our results indicate that exposure of the cells to a short pulse of ACTH can result in mRNA and enzyme induction as well as continued exposure of the cells to ACTH. In contrast, even a prolonged pulse of cAMP or adenylate cyclase stimulator, forskolin, is ineffective. These results require a reexamination of the previous hypotheses about the role of cAMP as the sole mediator of trophic hormonal effects on P450 gene induction. The inductive ability of a brief pulse of ACTH indicates that ACTH can rapidly initiate a series of reactions that result in enzyme induction many hours later.

MATERIALS AND METHODS

Bovine adrenal cortex cells were cultured following previous protocols (10, 17, 23), with some modifications: The adrenal cortex tissue was scraped from the capsule avoiding the glomerulosa layer that adheres to the capsule. The scrapings were cut to small pieces using a fine scissor, washed with Ham's F-12 medium, and incubated (1 g of tissue/10 ml of medium) in Ham's F-12 medium containing 10% fetal calf serum, 2 mg/ml collagenase (Sigma, Type IA), and 0.1 mg/ml DNase I (Boehringer Mannheim), for 1 h at 37 °C with gentle shaking. The suspension was flushed through a pipette several times and the undigested tissue was then allowed to settle for 1–2 min. The upper cell suspension was filtered through two layers of gauze and centrifuged at 100 × *g* for 5 min. The pelleted cells were washed twice by resuspension in F-12 medium (volume equal to the original suspension) and centrifugation at 100 × *g* for 5 min. During cell pellet resuspension, pipetting was avoided because it increased cell mortality probably by shearing cells that became fragile after digestion. The final cell pellet was resuspended in Dulbecco's modified Eagle's medium and Ham's F-12 (1:1) containing 12.5% horse serum, 2.5% fetal calf serum (Biolab, Israel), basic fibroblast growth factor (0.3 ng/ml medium; Boehringer Mannheim), penicillin (50 IU/ml), streptomycin (50 μg/ml), and mycostatin (5 IU/ml; antibiotic/antimycotic

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[1] The abbreviations used are: ACTH, corticotropin; Br, bromo.

solution from Biolab) at a density of 3×10^5 cells/ml and plated in 35- or 100-mm tissue culture dishes (Falcon Labware, Oxnard, CA). Cells were grown in tissue culture incubator with an atmosphere of 8% CO_2 and 92% air at 37 °C.

The medium was changed a day after plating. Subsequently, the culture was grown to confluence replacing the medium every 2 days. A day before the experiment, the medium was replaced with fresh medium without antibiotics and fibroblast growth factor. All experiments were done with cells maintained in Dulbecco's modified Eagle's medium and Ham's F-12 (1:1) containing 12.5% horse serum, and 2.5% fetal calf serum, except as specified in Fig. 4, legend. $ACTH_{1-24}$ (Organon Pharmaceuticals) and cAMP analogs (Sigma) were added in freshly prepared media.

For immunofluorescence studies, cells were plated on siliconized glass cover slips in 24-well tissue culture plates. Siliconization of the cover slips was necessary for cell adhesion. Cells did not adhere to cover slips coated with polylysine or serum. Immunofluorescence was performed essentially as described (24). The cells were observed in a Zeiss photomicroscope III equipped with a vertical fluorescence illuminator.

For Western blot analysis, cells were washed once with a cold solution of phosphate buffered saline (130 mM NaCl, 2.5 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.4)). The cells were then scraped and collected in Eppendorf tubes, lysed by freezing and thawing, and kept frozen at a concentration of about 1–2 mg protein/ml. Aliquots of this suspension were electrophoresed on polyacrylamide gels, and Western blots were prepared and reacted with antibodies and [^{125}I]protein A as described (25, 26). Purification of the enzymes and generation of the antibodies were performed in our laboratory (25).

Total cellular RNA was isolated as described (27) with one modification: the medium was decanted and the plates were washed once with cold phosphate buffered saline. The cells were scraped in 1.5 ml of phosphate buffered saline on ice. Scraping the cells in the original medium was frequently associated with RNA degradation. The yield of total RNA varied between 20 and 30 μg per 100-mm plate. For Northern blot analysis RNA was pooled from two to three plates for each determination. Northern blots were prepared using nylon membrane, GeneScreen (Du Pont-New England Nuclear), and reacted with ^{32}P-labeled cDNA probes as described (28, 29). The integrity and quantity of the RNA on each blot was checked by methylene blue staining of the blot (28) prior to any hybridization reaction. The cDNAs for adrenodoxin and $P450_{scc}$ were derived from clones generously provided by Dr. M. R. Waterman (12, 19). The cDNA for adrenodoxin reductase was cloned by us (29, 30).

The cells used for the cAMP assay were grown to confluence in 35-mm plates under the same conditions as those for enzyme induction. Cyclic AMP was measured using Gilman's protein binding assay (31). Protein was measured using a Bradford (32) reagent (Pierce Chemical Co.) with bovine serum albumin as standard. The bands on autoradiograms were quantitated using a Bio-Rad or a Molecular Dynamics computing densitometer. Cortisol was measured by direct radioimmunoassay using a solid-phase kit (Zer, Israel). All analytical measurements were done in duplicate.

RESULTS

Immunofluorescent Localization of Mitochondrial Cytochrome P450 System Enzymes—As the first step in our studies in cultured adrenal cortex cells we wanted to answer two questions: 1) Do the cultured cells represent a homogeneous population of cells each of which includes the steroidogenic enzymes? 2) Do the cells respond uniformly to stimulation with ACTH?

Previous immunofluorescence studies (18) indicate that as adrenocortical cells in primary culture multiply and reach confluence the immunofluorescent staining of $P450_{scc}$ in the mitochondria decreases but does not disappear. After stimulation with ACTH the immunofluorescent staining of $P450_{scc}$ increases uniformly in all cells (18). Figs. 1 and 2 show that similar to $P450_{scc}$, adrenodoxin and adrenodoxin reductase are also uniformly induced in all cells. However, a noticeable stimulation was not seen using $P450_{c11}$ antibodies (see below).

The intensity of immunofluorescent staining of all mitochondria within the same cell was very similar (Figs. 1 and 2). This contradicts previous reports (33, 34) which indicate that adrenodoxin and adrenodoxin reductase have differential distribution within mitochondria in a single adrenocortical cell.

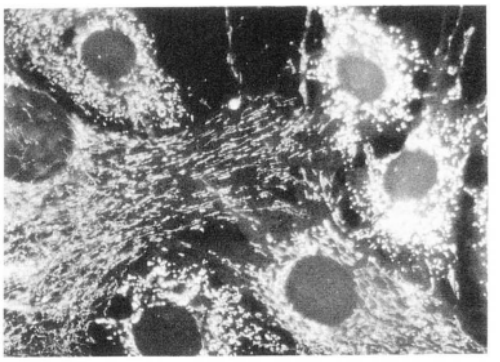

FIG. 1. **Immunofluorescent staining of mitochondria in adrenal cortex cells 9 days in culture and 48 h after addition of 1 μM ACTH.** The antibody used was against adrenodoxin reductase. Note that the nuclei are not stained. These cells were located at the periphery of a confluent area and hence show greater spread than those in confluent areas (compare with Fig. 2).

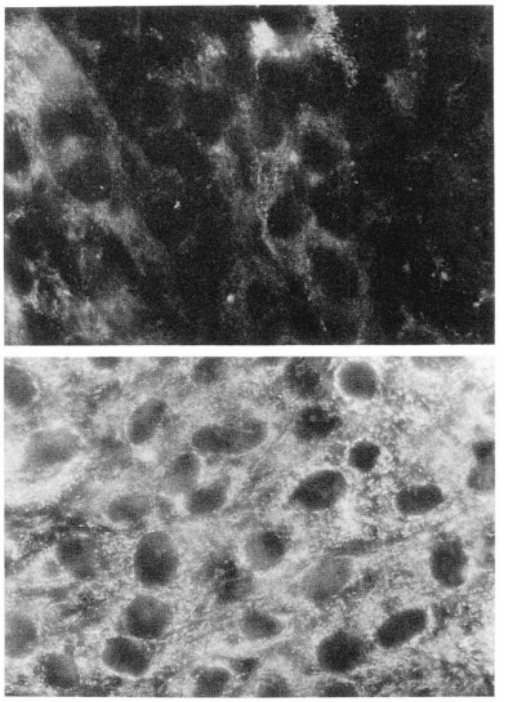

FIG. 2. **Immunofluorescent staining of mitochondria in adrenal cortex cells 9 days in culture with (*bottom*) and without (*top*) ACTH (1 μM) stimulation for 48 h.** The antibody used was against adrenodoxin. Both photographs were taken with a 30-s exposure. These cells were located in a completely confluent area and hence are tightly packed.

Time Course of Induction of Enzymes by ACTH—After observing that the cultured adrenal cortex cells represent a homogeneous population of steroidogenic cells and respond uniformly to ACTH, we determined the time course of induction of the four enzymes of the mitochondrial P450 system enzymes (Figs. 3 and 4). In culture medium containing serum, ACTH increased the levels of $P450_{scc}$, adrenodoxin, and adrenodoxin reductase but not that of $P450_{c11}$ (Fig. 4). In culture medium containing antioxidants, instead of serum, the levels of all four enzymes increased (Fig. 4). But this medium did not appear to be optimal for cell growth because invariably

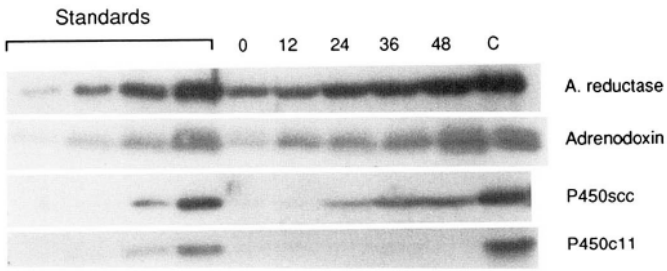

FIG. 3. **Western blot quantitation of ACTH induction of mitochondrial P450 system enzymes in adrenal cortex cells in culture.** The figure shows autoradiograms of Western blots of four separate protein gels reacted with the antibodies against the enzymes (25). In each gel the standards were 0.25, 0.5, 1, and 2 pmol of the indicated protein purified from bovine adrenal cortex (25). Each lane to the right of the standards contained 20 μg (for adrenodoxin only) or 30 μg total cell protein. The cells were grown to confluence and then treated continuously with 1 μM ACTH for the time indicated on *top* of the *lanes*, and collected by scraping. The *last lane* (*C*) included 5 μg of total protein from cells prepared just prior to plating. The medium was replaced after 24 h including fresh ACTH.

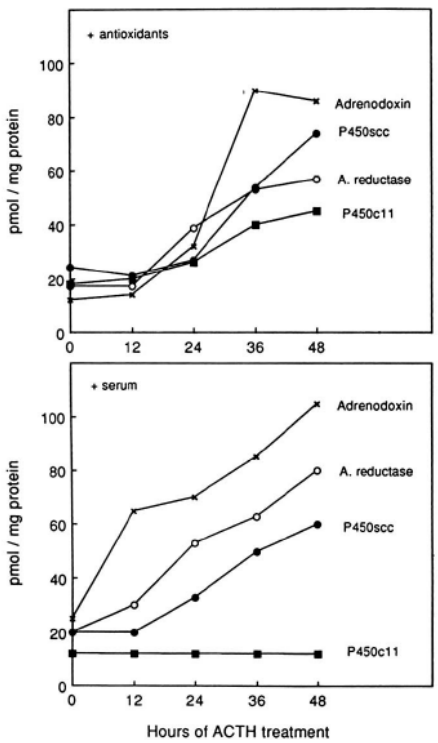

FIG. 4. **Time course of ACTH induction of mitochondrial P450 system enzymes in adrenal cortex cells in culture medium with serum or with antioxidants.** Cells were incubated with 1 μM ACTH for the times indicated on the *abscissa*, and then the cells were collected by scraping. *Top*, cells were grown in the same medium as in all other experiments but instead of serum the medium was supplemented with 100 μM ascorbic acid, 20 nM sodium selenite, 1 μM α-tocopherol, 20 nM insulin, and 50 μg/ml bovine serum albumin (35). The quantitation of the enzymes is based on densitometric scanning of autoradiograms of Western blots as shown in Fig. 3. The medium was replaced after 24 h including fresh ACTH.

the culture under these conditions showed shredding of what appeared to be cell fragments. Apart from $P450_{c11}$ the two different media compositions did not appear to have a major effect on the maximal levels of the enzymes (Fig. 4).

Except for adrenodoxin, the induction of the enzymes could be detected only 12–24 h after ACTH addition. The levels of all enzymes reached maximal levels in 36–48 h. Since in each gel for Western blot we run standards of purified enzymes we can quantitate the enzyme levels in molar amounts rather than in relative units of densitometric intensity. This permits comparison of the amounts and molar ratios of the enzymes in cells in different experiments and in cultured cells *versus* the adrenal cortex tissue. The maximal levels of the enzymes after induction was similar in different experiments with different cell preparations. The addition of fresh medium with ACTH after 24 h did not appear to significantly increase the maximal levels of the enzymes (compare Figs. 4 and 7).

The maximal levels of the enzymes in the cells (except that for adrenodoxin reductase) are lower than that in the total adrenal cortex tissue homogenate (25). This difference is even greater when the amounts of the enzymes in the cultured cells are compared with that in freshly isolated adrenocortical cells (Fig. 3). The levels of the enzymes in the freshly isolated cells are higher than that in bovine adrenal cortex tissue homogenate (25) probably reflecting the fact that the tissue homogenate contains many nonsteroidogenic cells and other protein components that decrease the amount of the enzymes when measured as pmoles of enzyme/mg of total protein.

Time Course of Induction of mRNAs by ACTH—The induction of $P450_{scc}$, $P450_{c11}$, and adrenodoxin mRNAs by ACTH in adrenal cortex cells has been already studied (12–15, 19). However, until the present report no study measuring both the amounts of the enzymes and the mRNAs was performed. Moreover, since the cDNA for adrenodoxin reductase was only recently cloned by us (29, 30) we wished to answer the following questions: 1) Is the induction of adrenodoxin reductase at protein level (Figs. 3 and 4) associated with an increase in the levels of its mRNA? 2) Are the time courses of mRNA induction for adrenodoxin reductase, adrenodoxin, and $P450_{scc}$ similar?

Northern blots indicate that 12 h after ACTH addition adrenodoxin reductase mRNA shows an increase (Fig. 5). Comparison of the time courses of induction of the mRNAs and the three proteins reveals two major differences (Figs. 4 and 5): 1) Adrenodoxin shows the fastest increase in both mRNA and protein (in serum supplemented medium), mRNA reaching maximal levels in 6 h (Fig. 5). 2) For all three proteins, but especially for adrenodoxin reductase and $P450_{scc}$, the increase in protein lags behind the increase in mRNA by many hours. These differences were consistently observed in independent experiments.

Time Course of Induction of cAMP by ACTH—Since cAMP has been suggested as the intracellular mediator of the effects of ACTH on P450 gene expression we measured the levels of cAMP in the cells after ACTH stimulation. After ACTH addition, the intracellular level of cAMP increases rapidly, reaching maximal levels within 5 min, and then decreases gradually over 120 min (Fig. 6). In contrast, the level of cAMP in the medium increases and reaches a plateau (Fig. 6). It is noteworthy that the sum of the cAMP levels in the cells and in the medium remain nearly constant in the time course studied. This may indicate that the cAMP level in the medium reflects cAMP that leaks out of the cells to the medium. The absolute amounts of cAMP measured by us in the medium are in the same range (about 2-fold higher) as previous measurements of cAMP secretion to the medium (18).

The experiment shown in Fig. 6 was carried out in the absence of a phosphodiesterase inhibitor in the medium. When ACTH stimulation of the cells was carried out in the presence of 0.1 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, the shape of the intracellular levels of cAMP looked virtually identical to that in Fig. 6, but the maximal level of cAMP was higher at about 3000 pmol/mg of protein (data not shown). Thus, the presence of phosphodi-

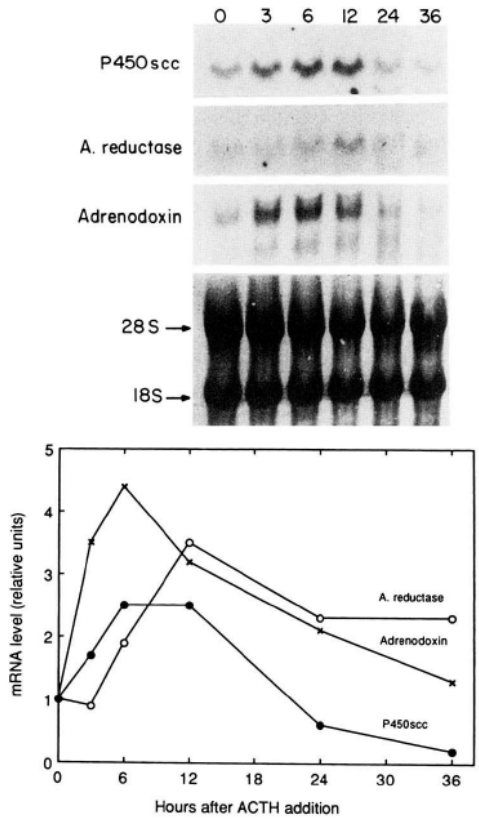

FIG. 5. **Time course of ACTH induction of adrenodoxin reductase, adrenodoxin, and $P450_{scc}$ mRNAs in adrenal cortex cells in culture.** Total RNA was isolated from cells incubated with 1 μM ACTH for the times indicated, run on 1.2% agarose gel (30 μg of total RNA/lane) and then blotted onto GeneScreen membrane. The same blot was reacted with three cDNAs in the following order: adrenodoxin reductase, $P450_{scc}$, and adrenodoxin. Prior to each reprobing, the blot was exposed to film to verify that previous hybridization would not interfere. *Top,* photographs of the autoradiograms and 28 S and 18 S ribosomal RNAs on blot stained with methylene blue; *bottom,* densitometric quantitation of the autoradiograms. The density of the time points is expressed relative to 0 time control which was taken as 1.

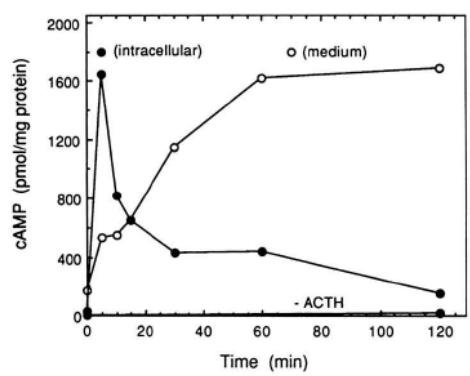

FIG. 6. **Time course of ACTH induction of cAMP synthesis in adrenal cortex cells in culture. Cells were incubated with 1 μM ACTH for the time indicated on the abscissa.** At the end of the incubation the medium was collected and brought to 50 mM sodium acetate (pH 4.0), and 0.1 mM 3-isobutyl-1-methylxanthine, for measurement of the cAMP amount in the medium. 2 ml of 50 mM sodium acetate (pH 4.0), and 0.1 mM 3-isobutyl-1-methylxanthine was then added to the plates and the plates were floated on boiling water for 5 min. The cells were then scraped and collected with the solution. The amount of cAMP in this solution was used to calculate the intracellular cAMP levels shown on the ordinate.

esterase inhibitor in the medium during stimulation did not alter the time course of changes in intracellular levels of cAMP.

Induction of Enzymes and mRNAs by Pulses of ACTH and cAMP—ACTH is secreted in pulses and its blood levels remain high for only a few hours (22). Hence, we wanted to examine if treatment of the cells for a short period simulating the physiological situation is capable of causing induction similar to that observed with continuous stimulation of the cells as in experiments in Figs. 4 and 5. We also examined the effects of cAMP analogs and an adenylate cyclase stimulator forskolin in parallel experiments.

Exposure of the cells to a brief pulse of ACTH as short as 30 min was able to effect maximal induction of adrenodoxin reductase, adrenodoxin, and $P450_{scc}$ 36 h after the addition of ACTH (Fig. 7 and data not shown). In these experiments we paid particular attention to remove ACTH by three successive changes of medium. As a further control, in one experiment we incubated cells with a medium exposed to a plate that was incubated with ACTH. Cells exposed to this control "wash medium" did not show increased levels of the enzymes. This indicated that the washes were effective and did not leave residual ACTH that could be released into the medium. However, the washes certainly would not remove receptor bound ACTH which most likely undergoes internalization like other peptide hormones that bind to cell surface receptors.

In contrast to ACTH, a pulse of dibutyryl cAMP as long as 2 h was not capable of inducing the enzymes under the same conditions. Yet, confirming previous observations for adrenodoxin and $P450_{scc}$ (18), continuous presence of this agent for 36 h indeed increased the levels of these enzymes, as well

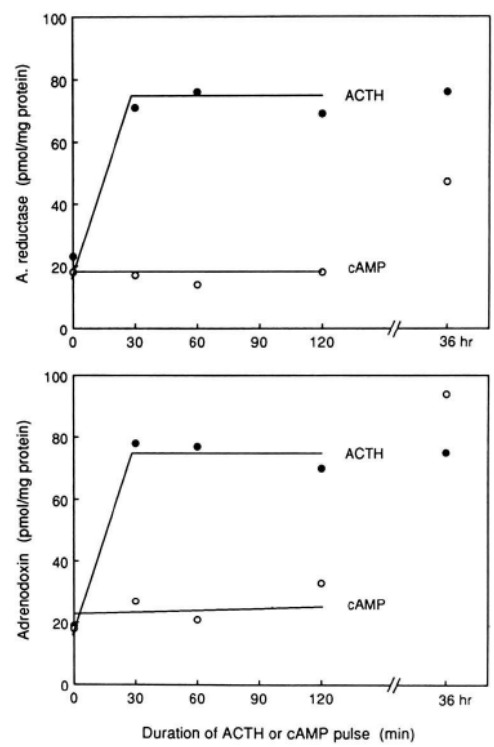

FIG. 7. **The effect of pulses of ACTH and dibutyryl cAMP on adrenodoxin reductase and adrenodoxin levels in adrenal cortex cells in culture.** Cells were incubated with 1 μM ACTH or 1 mM dibutyryl cAMP for the times indicated on the *abscissa.* The medium was then removed, and the plates were washed with medium twice. Fresh medium (without the stimulating agent) was then added and the cells were incubated for 36 h. Thus, each point shows the amount of enzyme in cells 36 h after the initiation of the experiment with the addition of the tested agent.

as adrenodoxin reductase, to levels obtained with ACTH (Fig. 7 and data not shown). For these experiments the 2-h maximal period of pulse was taken because after ACTH stimulation the intracellular levels of cAMP drop to very low levels after 2 h (Fig. 6). Similarly, a pulse of 8-Br-cAMP as long as 12 h also was not capable of inducing adrenodoxin (Fig. 8). As a control for the activity of this agent, the increase in cortisol secretion induced by it could be detected within a short time (Fig. 8). Additional experiments with forskolin, in an experiment parallel to that for dibutyryl cAMP, yielded virtually identical results indicating that intracellular elevation of cAMP for periods of a few hours is incapable of inducing the expression of the genes.

To examine the effect of pulses of ACTH and 8-bromo-cAMP on the mRNAs we exposed the cells again to a pulse of either agent for increasing intervals and isolated mRNA 12 h after addition of the agents. The 12-h period was taken because this was the time range where the maximal level of the $P450_{scc}$ mRNA was observed (Fig. 5). Extending the observations on enzyme levels, a pulse of ACTH was again seen to be sufficient to increase the mRNA to maximal levels, as well as continuous exposure of the cells to ACTH (Fig. 9). In striking contrast, a pulse of 8-Br-cAMP as long as 12 h was without an effect on the levels of $P450_{scc}$ mRNA (Fig. 9). The level of cortisol in the medium of ACTH or 8-Br-cAMP-treated cells was very similar (26, 85, 128 ng/ml for ACTH, and 30, 69, 98 ng/ml for 8-Br-cAMP, respectively, at 2, 4, and

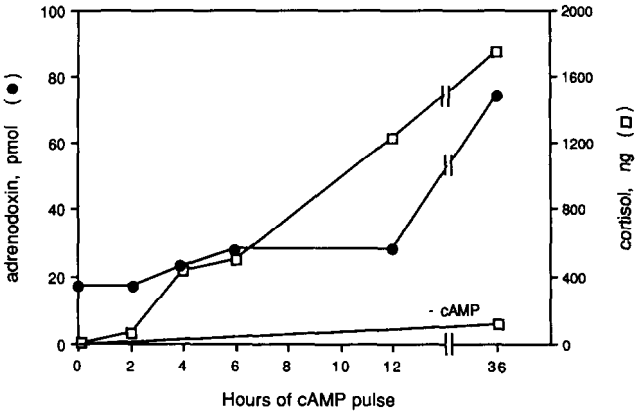

FIG. 8. **The effect of pulses of 8-bromo-cAMP on adrenodoxin levels and cortisol secretion in adrenal cortex cells in culture.** Cortisol was measured in the medium and expressed as per mg of protein cells in culture. For other details see Fig. 7, legend.

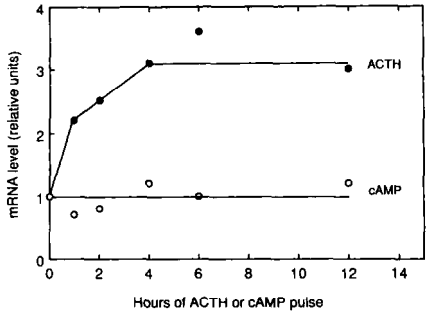

FIG. 9. **The effect of pulses of ACTH and 8-bromo-cAMP on the levels of $P450_{scc}$ mRNA in adrenal cortex cells in culture.** Cells were incubated with 1 μM ACTH or 1 mM 8-bromo-cAMP for the times indicated on the *abscissa*. The medium was then removed, and the plates were washed with medium twice. Fresh medium (without the stimulating agent) was then added and the cells were incubated for 12 h. Each point shows the relative amount of mRNA in cells 12 h after the initiation of the experiment with the addition of the tested agent.

6 h after addition of ACTH or 8-Br-cAMP). This again indicates that 8-Br-cAMP was as effective as ACTH in induction of cortisol secretion, despite its lack of effect on mRNA levels.

DISCUSSION

ACTH Induces All Three Enzymes of the Mitochondrial P450 System at Both mRNA and Protein Levels—The results of this study indicate that ACTH can increase the levels of all four enzymes of the mitochondrial P450 system enzymes in adrenal cortex cells in culture (Fig. 4). Previous studies indicated that ACTH can increase the synthesis of $P450_{scc}$, $P450_{c11}$, and adrenodoxin, and also enhance the transcription of the genes coding for these enzymes in adrenal cortex cells (6, 14). The present results on adrenodoxin reductase complete this picture by showing that all three components of the mitochondrial P450 system are increased by ACTH at the level of both mRNA and protein. However, a comparison of the time courses of induction of the mRNAs and the three proteins (Figs. 4 and 5) reveals some major differences as detailed below.

$P450_{scc}$ and adrenodoxin reductase show similar time courses at both protein and mRNA level. But, adrenodoxin protein and mRNA show significantly earlier increases than the other two enzymes (in serum-supplemented medium). Since these differences are consistently observed in the same experiments these results indicate that there may be differences in the expression of the genes coding for these proteins.

Is ACTH Induction of the Enzymes Only a Result of Enhancement of mRNA Synthesis?—The mRNA levels of all three enzymes increase and reach a peak within 3–12 h and then drop between 12 and 24 h after ACTH addition (Fig. 5). In contrast, the increase in the protein levels of these enzymes is much slower especially for $P450_{scc}$ and adrenodoxin reductase which show an increase only nearly 24 h after ACTH addition (Fig. 4). The time courses of increase in $P450_{scc}$ mRNA (Fig. 5) is consistent with previous reports in bovine adrenocortical cells (12, 14, 15). A study in human fetal adrenocortical cells showed a much slower (>24 h) induction of the $P450_{scc}$ mRNA (9). In bovine cells the changes in $P450_{scc}$ mRNA levels was previously followed up to 12 h (14, 15) or 60 h (12). The latter study did not report a decrease in $P450_{scc}$ mRNA as we observe (Fig. 5) but then in this study medium was replaced after 24 h including fresh ACTH (12) whereas we did not do so. Since no previous study measured the increase in both protein and mRNA for these enzymes, the results here can not be compared directly with previous reports. However, previously the peak times for the synthesis of $P450_{scc}$, adrenodoxin, and for $P450_{scc}$ activity were reported as between 36 and 48 h after ACTH addition (10, 16–18), which is again consistent with our findings.

The very significant delay in the time courses of ACTH induction of the proteins *versus* the mRNAs (Figs. 4 and 5, and other studies noted above) reveals a significant gap in our understanding of the processes that ultimately result in increased enzyme levels. Previously no attention was drawn to this lack of close parallelism in the time courses of proteins and mRNAs. At present we do not know the reason for the lag between the increase in the mRNAs and the proteins. Previously published (12, 14, 15) detailed time courses of induction of mRNAs were based on quantitation of "Slot-blot" experiments, whereas our analyses are based on quantitation of the mature mRNA by Northern blots of RNA gels (Fig. 5). Thus, we clearly see that the increase in mRNA is observed at the level of the mature mRNA and that during the time course of mRNA induction there does not appear to

be a change in the mRNA processing. However, despite the fact the mRNA levels reach a peak at 12 h, prior experiments with *in vitro* translation of mRNA from ACTH-stimulated cells do not show any detectable increase in the synthesis of $P450_{scc}$ 12 h after ACTH addition (10, 16). Overall, these results indicate that there may be a delay in the translation of the mRNAs to protein and that the increase in mRNA is not directly associated with increased translation to protein. Whether ACTH regulates the translation of the mRNAs to protein, and what is responsible for the delay in the translation of mRNA to protein, remain as open questions.

The Protective Effect of Antioxidants Is Specific to $P450_{c11}$—Previous studies (11) indicate that ACTH induction of $P450_{c11}$ can be inhibited by the product of this enzyme, cortisol. Accumulation of cortisol in the medium can cause degradation of $P450_{c11}$. This process can be inhibited by the addition of antioxidants to the medium (11). Our results confirm these findings and further indicate that the levels of other enzymes are not significantly effected by antioxidants (Fig. 4). Moreover, ACTH stimulation of the cells in the absence or presence of serum indicates that serum does not effect the induction of the enzymes (Fig. 4). The observed specificity of the effect of antioxidants only on $P450_{c11}$ (Fig. 4), strengthens the previous hypothesis that the association of $P450_{c11}$ with its product is responsible for its degradation by a free radical reaction (11). A previous study could not confirm this finding of inhibition of $P450_{c11}$ induction (17). This was ascribed to the possibility of differences in growth or initial plating conditions which may cause the variability in results (17).

Differences in the Expression of the Enzymes in Cultured Cells versus Tissue—In our studies we quantitate the induction of the enzymes in molar amounts, whereas, previous studies have generally measured this process by *in vitro* synthesis or translation of the induced mRNAs yielding only relative measures of densitometric intensity. Our measurements permit comparison of the levels of the enzymes in cultured cells and in normal adrenal cortex and reveal two major differences: the induced levels of the enzymes in the cultured cells are several- to many-fold lower than that in normal cells in tissue, and the molar ratios of the enzymes are different in cultured cells and tissue (compare Figs. 3 and 4 and Ref. 25). Thus, qualitatively the cultured cells show a response to ACTH similar to that of cells in tissue, *i.e.* ACTH induces genes in cultured cells the *in vivo* expression of which are also dependent on ACTH. However, the quantitative measurements of this response in cultured cells are different from the normal tissue. These differences probably reflect different efficiencies in transcription of the mRNA and its translation. The gross alterations in the intracellular organization of the cultured cells, and the lack of a normal contact with cells and extracellular matrix (36) may be some of the reasons responsible for these differences.

An ACTH Pulse Can Induce the Enzymes as well as Continuous Stimulation—The experiments with continuous ACTH stimulation consistently shows that the time course of induction of the enzymes and their mRNAs is a slow process the results of which can be detected only after many hours (Figs. 4 and 5). The type of experimental protocol where the cells are continuously stimulated does not answer the following questions: 1) Is the ACTH stimulation of the mRNA and protein synthesis, the cumulative result of a process that has to be continuously stimulated, or is it intrinsically a slow process that once started requires time for its full expression? 2) Since the blood levels of ACTH remain high for only several hours a day (22), is the simulation of this situation *in vitro* capable of inducing the enzymes?

In an attempt to answer the questions above we exposed the adrenocortical cells to a single pulse of ACTH and continued to incubate the cells in the absence of ACTH. Our results invariably showed that exposure of the cells to ACTH for a few hours or less is sufficient for maximal induction of the enzymes many hours later (Figs. 7 and 9). These results indicate that 1) the ACTH induction of the enzymes is an intrinsically slow process that does not require continuous stimulation and 2) an ACTH pulse, simulating physiological situation can induce this process as well as continuous stimulation.

Is cAMP the Sole Mediator of ACTH Induction of the Mitochondrial P450 System Enzymes?—ACTH, like other peptide hormones, bind to specific cell membrane receptors and activate a number of intracellular signaling systems, *e.g.* cAMP, and phosphoinositides (37–40). A series of studies suggested that cAMP is the mediator of ACTH in inducing steroidogenic enzymes (6). This conclusion was based mainly on two types of observations: 1) ACTH can stimulate a continuous increase in cAMP levels in the culture medium; and 2) cAMP analogs can induce the enzymes to the same levels as ACTH stimulation (6, 18). The results of our experiments with continuous stimulation for 36–48 h confirm these results quantitatively. However, measurement of cAMP levels in the cell, in addition to those in the medium, and the results of the experiments with exposure of the cells to a pulse of ACTH or cAMP analogs, provide a different view as detailed below.

After ACTH stimulation, cAMP level in the cell shows a very steep increase but decreases nearly as sharply in about an hour (Fig. 6). In understanding the role of cAMP as a second messenger, the relevant measure is its concentration in the cell and not in the medium. The cAMP in the medium appears to reflect not new synthesis in the cell but rather leakage of previously synthesized cAMP outside of the cell (see "Results" and Fig. 6). Our results in the adrenocortical cells in culture are consistent with previous results in different systems (*e.g.* 38, 39, 41, 42). We used a concentration of ACTH necessary for maximal stimulation of the cells (18) which is higher than its physiological levels. However, the time course of decrease in cAMP levels have been observed to be similar over a wide range of a trophic hormone concentration (42).

In contrast to the results observed with ACTH, exposure of the cells for several hours to cAMP analogs or forskolin, do not increase enzyme or mRNA levels. The stimulation of steroid secretion by the cAMP analogs indicates that during these "pulse" experiments they penetrate into the cell and activate at least some intracellular processes (Fig. 8). This observation makes it rather unlikely that the lack of effect is a result of a "bioavailability problem" for the added agents. With these results, we have no doubt that elevation of intracellular cAMP, either by cAMP analogs or activation of endogenous adenylate cyclase by forskolin, can induce the enzymes and their mRNAs only after unphysiologically long exposure of the cells to these agents.

Recently, the activation of a fusion gene containing the 5′-flanking region of $P450_{scc}$ gene by cAMP analogs and forskolin has been demonstrated in a mouse adrenocortical cell line (43). In these experiments significant induction is observed only after continuous stimulation for 24 h (43), a time interval similar to that observed by us and others in the primary cultures. In a recent study using different constructs of $P450_{scc}$ gene rapid expression of the reporter gene has been demonstrated in response to forskolin stimulation (44). A cAMP responsive regulatory element has been detected and charac-

terized in the $P450_{c11}$ gene; yet similar sequences have not been observed in other enzyme genes that are coordinately regulated (20, 44). Interestingly, the induction of $P450_{c11}$ mRNA by cAMP analogs can be observed within a few hours (13, 20), a time that is shorter than that observed for $P450_{scc}$ gene expression.

Overall, three major experimental observations indicate that cAMP does not function as the sole intracellular mediator of ACTH induction of the enzymes: 1) ACTH levels in the blood remain high only for several hours (22). 2) ACTH stimulation of cAMP synthesis highly increases intracellular cAMP levels for only a brief interval, after which the system is desensitized. 3) The enzymes and their mRNAs can be induced by stimulation of the cells with a 1–2-h long pulse of ACTH, but not by elevation of intracellular cAMP levels for the same or even longer duration, either by forskolin or soluble cAMP analogs. Given these observations we conclude that the induction of the enzymes by continuous stimulation of cells for >12 h with various agents that elevate intracellular cAMP levels is a pharmacological, but not a physiological effect.

It should be emphasized that the present observations do not eliminate a role for cAMP, but raise problems in accepting cAMP as the sole mediator of ACTH . In mouse adrenocortical cell lines, a defective cAMP-dependent protein kinase interferes with the basal and cAMP-stimulated expression of $P450_{c11}$ and $P450_{scc}$ (45). Although, the cAMP stimulation of these cells was done for extended periods, the effects nonetheless provide evidence for the involvement of cAMP in the regulation of expression of these enzymes (45). Yet, studies on the expression patterns of different steroidogenic enzymes during developmental stages and in *in vitro* systems have rightly led to the conclusion that "regulation of steroid hydroxylase gene expression is complex and multifactorial involving cAMP-dependent and -independent mechanisms" (6).

Our observations of the inductive ability of a brief pulse of ACTH indicates that ACTH can rapidly initiate a series of reactions that result in enzyme induction many hours later. Some of these early events still remain to be identified. On the basis of the present findings, the intracellular agent(s) that mediate ACTH induction of enzymes would be expected to fulfil a new criteria: the ability to act in a short pulse as ACTH. The conclusions of this study may also be applicable to the actions of the trophic hormones of other steroidogenic tissues.

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Note Added in Proof—Two recent studies report electron microscopic localization of adrenodoxin in adrenal cortex (46) and ovarian granulosa cells (47).

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