Regulation of Δ^5-3β-Hydroxysteroid Dehydrogenase-Isomerase Activity in Adrenocortical Cell Cultures by Adrenocorticotropin

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SUMMARY: Δ^5-3β-Hydroxysteroid dehydrogenase-isomerase activity was found to decay in primary cultures of normal rat adrenocortical cells maintained in the absence of adrenocorticotropin for more than 7 days. Physiological concentrations of adrenocorticotropin induced the enzyme complex with a lag period of about 4 hours. Studies with actinomycin D and cycloheximide suggested that both RNA and protein synthesis are required for the induction of steroid dehydrogenase-isomerase activity.

INTRODUCTION

The major functions of adrenocorticotropin (ACTH)* are the acute stimulation of steroidogenesis in the adrenal gland and the long term regulation of the growth and maintenance of the adrenal cortex. Recent studies of the long term actions of ACTH on primary monolayer cultures of normal rat adrenocortical cells have shown that the hormone inhibits the proliferation of adrenocortical cells with a concomitant stimulation of steroidogenesis (1). Studies with rats deprived of endogenous ACTH by the administration of specific antibodies to ACTH have shown that the trophic action of the hormone is solely concerned with the maintenance of the steroidogenic capacity of the adrenocortical cells (2,3). We have investigated the trophic actions of ACTH in primary monolayer cultures of normal rat adrenocortical cells by studying the induction and maintenance

* Abbreviations used: 3β-HSD, Δ^5-3β-Hydroxysteroid dehydrogenase-isomerase; ACTH, adrenocorticotropin; FCS, fetal calf serum; RIA, radioimmunoassay.

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of Δ^5-3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD). This enzyme complex is required for the conversion of pregnenolone to progesterone, a key intermediate in the biosynthesis of corticosteroids in the adrenocortical cell (4,5). The results show that monolayer cultures of rat adrenocortical cells are of great value for investigating the molecular mechanisms involved in the induction of specific enzymes by a polypeptide hormone.

MATERIALS AND METHODS

Highly purified ACTH was prepared in this laboratory (6). ^{3}H-progesterone was obtained from New England Nuclear; all other chemicals from Sigma. Medium 199 containing D-valine instead of L-valine was obtained from the Cell Culture Facility of the University of California at San Francisco. Antibiotics and fetal calf serum (FCS) were purchased from GIBCO and tissue culture dishes were from Falcon plastics. FCS was dialyzed against two changes of phosphate buffered saline, pH 7.4 (8.00 g NaCl, 0.20 g KCl, 0.20 g KH_2PO_4, 0.15 g Na_2HPO_4 per liter) for 24 hr at 4°

Cell cultures: Adrenocortical cells were isolated from the decapsulated adrenals of male Sprague-Dawley rats (250-300 g) as previously described (1). The cells were suspended in medium 199-D-Val containing 10% dialyzed FCS, 50 μg/ml gentamycin, and 10 U/ml mycostatin and plated in multiwells or 35 mm dishes at densities indicated for each experiment. The medium was changed every 48 hr.

3β-HSD assay: The enzyme activity was monitored by both histochemical staining of the cells (7) and by measuring the conversion of exogenous pregnenolone to progesterone as described by Solomon and Sherman (8). The cells were incubated with pregnenolone for 2-6 hr and the formation of progesterone was measured by specific radioimmunoassay using antiserum raised in rabbits against 11α-hydroxy progesterone hemisuccinate-bovine serum albumin conjugate (9). The cross reaction of different steroids with the progesterone antiserum was found to be: corticosterone, 0.76%; pregnenolone, 0.04%; testosterone, 0.02%; deoxycorticosterone, 0.01%; dehydroepiandrosterone, 0.008%; cortisol, 0.004%; aldosterone, 0.002%; estradiol and estriol, <0.001%.

RESULTS

Gilbert and Migeon (10) showed that fibroblast growth is arrested in D-valine media owing to the lack of D-amino acid oxidase. We have found that rat adrenocortical cells can be maintained in D-valine medium without fibroblast proliferation provided the FCS is dialyzed to remove any L-valine present in the serum. The adrenocortical

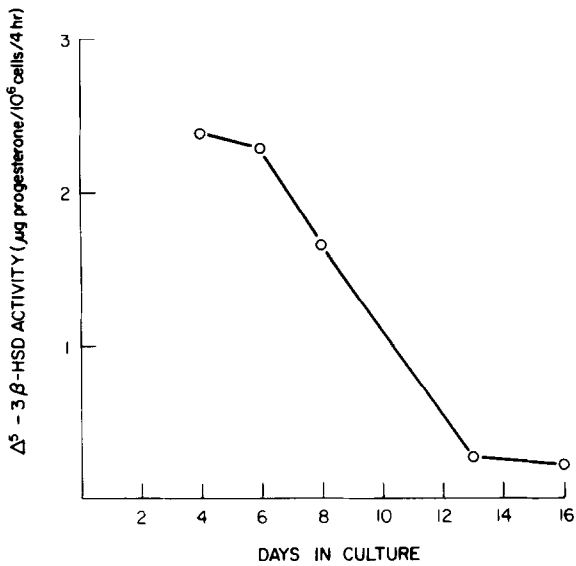

Figure 1. Decay of 3β-HSD activity in adrenocortical cell cultures. Cells were plated at a density of 1 x 10^5 cells/cm^2 in multiwells as described under methods. At different times the medium was removed and the wells were washed. Fresh medium was added followed by pregnenolone (8 μg in 0.01 ml ethanol). The cultures were incubated at 37° for 4 hr and then an aliquot of medium was assayed for progesterone by RIA. The points are the means of analyses of triplicate cultures.

cells from adult rats remained functional for a period of 7 days in culture as shown by the rapid production of corticosterone in response to ACTH, as well as the histochemical staining for 3β-HSD activity (unpublished observation). Cells maintained in the absence of ACTH for another 7 days stained poorly for 3β-HSD, whereas cells kept in the presence of ACTH during the second week of culture stained strongly for the enzyme. The decay of 3β-HSD in adrenocortical cell cultures in the absence of ACTH was followed by the more sensitive procedure of monitoring the conversion of pregnenolone to progesterone by radioimmunoassay (8). 3β-HSD activity remained at a steady high level during the first 6 days of culture and decayed to about 50% of the initial activity by day 8 (Fig. 1). There was

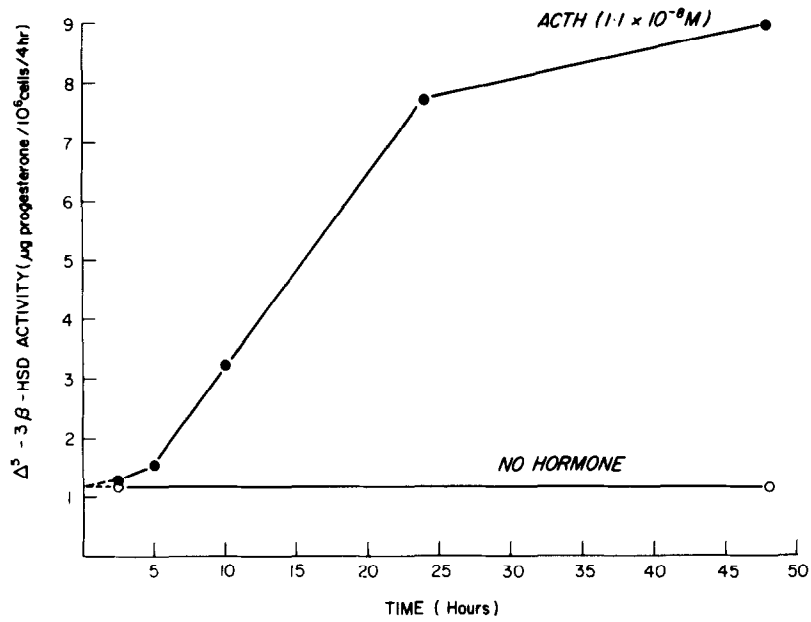

Figure 2. Kinetics of induction of 3β-HSD activity by ACTH. Cells were plated in 35 mm dishes at a density of 3 x 10^5 cells/cm^2. After 14 days in culture, ACTH (1.1 x 10^{-8} M) was added. At the times indicated the cells were washed and incubated with pregnenolone for 4 hr as described under Figure 1. Progesterone was assayed by RIA. The points are the means of analyses of triplicate cultures.

less than 15% of the initial 3β-HSD activity detectable in the cultures after 13 days.

Addition of ACTH to cultures maintained in the absence of the hormone for 14 days resulted in the induction of 3β-HSD activity. The kinetics of induction of the enzyme activity by ACTH is shown in Fig. 2. There was a lag period of about 4 hr before any significant increase in enzyme activity could be detected. Maximal increase in enzyme activity was observed 24 hr after the addition of ACTH. The induction of 3β-HSD activity was found to be a function of the concentration of ACTH (Table 1). After a 48 hr induction with different concentrations of ACTH, incubation of control and ACTH-stimulated cultures with pregnenolone for 2 and 6 hr showed that a 3.2 and 3.5 fold stimulation of enzyme activity,

Table 1

Induction of 3β-HSD Activity in Adrenocortical Cell Cultures

Additions	3β-HSD Activity (μg progesterone/10^6 cells)	
	2 hours	6 hours
None	0.97 ± 0.37	1.85 ± 0.19
ACTH (0.22 nM)	3.19 ± 0.64	6.54 ± 0.37
ACTH (2.2 nM)	4.09 ± 0.25	7.46 ± 1.05
ACTH (22 nM)	5.26 ± 0.58	7.20 ± 0.78

Adrenocortical cells were plated in 35 mm dishes at a density of 3×10^5 cells/cm^2 as described under Methods. After 9 days in culture, ACTH was added at the concentrations shown. 48 hr after the addition of ACTH, the cells were incubated with pregnenolone as described in the legend to Fig. 1 and progesterone production was monitored by RIA at 2 and 6 hr. The values are the mean ± SE of analyses of triplicate cultures.

respectively, was produced by 0.22 nM ACTH. Under the same conditions maximal stimulation (4-5 fold) was caused by 2.2 nM ACTH. High concentrations of other basic polypeptides such as β-endorphin (3×10^{-7} M) and fibroblast growth factor (10^{-7} M) failed to induce the enzyme activity.

The effects of inhibitors of RNA and protein synthesis on the induction of 3β-HSD by ACTH was examined (Table 2). Actinomycin D (0.02 μg/ml) completely inhibited the induction of the enzyme by ACTH but had little effect on the basal 3β-HSD activity. Cycloheximide (1.5 μg/ml), on the other hand, caused significant inhibition of both the unstimulated and ACTH-induced enzyme activity. Basal 3β-HSD activity was inhibited 50% and the induction by ACTH was inhibited 62%. High concentrations of dibutyryl cAMP (100 μg/ml) also caused the induction of 3β-HSD and cycloheximide blocked the

Table 2

Effects of Actinomycine D and Cycloheximide on the Induction of 3β-HSD by ACTH

Additions	3β-HSD Activity (μg progesterone/10^6cells/4 hr		
	No. hormone	ACTH (0.22 nM)	ACTH (2.2 nM)
None	8.45 ± 0.52	23.6 ± 1.94	33.0 ± 2.0
Actinomycin D (0.02 μg/ml)	6.06 ± 0.78	7.2 ± 0.90	8.9 ± 0.90
Cycloheximide (1.5 μg/ml)	4.43 ± 0.48	8.8 ± 1.33	12.0 ± 0.93
Dibutyryl cAMP (100 μg/ml)	32.0 ± 1.73		
Dibutyryl cAMP (100 μg/ml) + cycloheximide (1.5 μg/ml)	9.3 ± 0.63		
8-Br cGMP (100 μg/ml)	10.4 ± 0.64		

Adrenocortical cells were plated in multiwells at a density of 1 x 10^5 cells/cm^2. After 12 days in culture, ACTH and the cyclic nucleotides were added with or without the inhibitors. The medium was removed after 24 hr, the cells were washed with fresh medium and incubated with pregnenolone for 4 hr as described under Fig. 1. Progesterone production was assessed by RIA. Values are the mean ± SE of analyses of triplicate cultures.

action of cAMP. There was no significant induction of enzyme activity by 8-Br-cGMP (100 μg/ml).

DISCUSSION

It is known that ACTH is able to prevent the decay of steroidogenic capacity in hypophysectomized animals. Kimura (11) observed a decay of the cholesterol side-chain cleaving activity after hypophysectomy which was restored by the administration of ACTH. Purvis et al. (12) found that the concentrations of electron-transport carriers involved in the steroid hydroxylation reactions of the

adrenal cortex (NADPH-cytochrome P-450 pathway) are under the influence of ACTH. O'Hare and Neville (13) examined the metabolism of ^{3}H-pregnenolone by adult rat adrenocortical cells in culture and concluded that in the absence of ACTH, 21-hydroxylase activity was almost completely lost and 11β- and 18-hydroxylases were reduced. The 3β-HSD was reported to persist at a substantial level.

The results presented here clearly show that 3β-HSD activity decays in cultures of normal rat adrenocortical cells maintained in the absence of ACTH. The enzyme complex was readily induced by physiological concentrations of the hormone. The induction of 3β-HSD by ACTH was selectively inhibited by very low concentrations of actinomycin D suggesting a critical requirement for RNA synthesis in the induction process. There was little selective inhibition of the induction of the enzyme by cycloheximide. This inhibitor of cytoplasmic protein synthesis affected both basal and ACTH-induced 3β-HSD to nearly the same extent. These results suggest that ACTH may regulate 3β-HSD directly or indirectly at the transcriptional level.

It is apparent that monolayer cultures of normal adrenocortical cells are a very convenient experimental system for investigating the molecular mechanisms involved in the long term regulatory actions of ACTH. The synthesis of various enzymes in the steroidogenic pathway can be induced by the introduction of the hormone and turned off by withdrawal of the hormone. Further details of the induction of 3β-HSD as well as other steroidogenic enzymes and the temporal relationships of the induction of these enzymes are currently under study.

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