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Regulation of Adrenal Function

INHIBITION OF STEROIDOGENESIS IN RAT ADRENAL CORTEX CELLS BY A THREONINE ANALOGUE

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Summary—We have investigated the ability of amino acid analogues of serine and threonine to inhibit the increase in steroidogenesis elicited by addition of ACTH or cAMP in cells isolated from the rat adrenal cortex. We have found that the serine analogues, D, L-isoserine, α-methyl-D, L-serine and L-homoserine, are almost totally ineffective in inhibiting this process but that the threonine analogue, D, L-β-hydroxynorvaline, at a concentration of 300 μM inhibits stimulated steroid hormone biosynthesis by *ca* 95%, while inhibiting overall protein synthesis by only *ca* 40%. This inhibition was found to occur in a dose-dependent manner and to be reversible by a stoichiometric concentration of threonine. These studies suggest that β-hydroxynorvaline is functioning as a threonine analogue in our experimental system. Both the onset of inhibition by analogue and reversal of this inhibition by the natural amino acid occurred rapidly, without detectable lag. Since results obtained using cAMP as stimulant parallel those obtained using ACTH, the inhibitory effect of the analogue seems to occur subsequent to the synthesis of cAMP. Additionally, the analogue does not inhibit the conversion of pregnenolone to corticosterone, suggesting the site of action of analogue occurs prior to the synthesis of pregnenolone from cholesterol. Thus, the analogue may be exerting its effect on a protein that is synthesized subsequent to ACTH addition and is important in the acute phase of stimulated steroid hormone biosynthesis. Further, since ACTH action on adrenal cortex cells causes the activation of protein kinase A, which phosphorylates serine and threonine residues, it is possible that the effect of the analogue is to prevent the phosphorylation of a newly-synthesized protein.

INTRODUCTION

The peptide hormone ACTH acts on adrenal fasciculata cells to trigger a cascade of events which lead within 3–5 min to a 10 to 15-fold increase in the rate of synthesis of glucocorticoids [1,2]. ACTH, upon binding to its receptor [3] on the plasma membrane of these cells, stimulates adenylate cyclase, resulting in elevated membrane-associated cAMP levels [4]. These increased amounts of cAMP activate protein kinase A [4], which in turn phosphorylates a number of proteins presumably needed to cause increased steroidogenesis either in the acute, i.e. within minutes, phase of the response or in the longer, trophic response. Thus, protein phosphorylation is assumed to be a key regulatory event in stimulated steroid synthesis.

One of the proteins phosphorylated is cholesterol esterase, which releases free cholesterol by the hydrolysis of cholesterol esters stored in lipid droplets in the cytosol of the cell [5,6]. The released cholesterol is then transported, possibly via cytoskeletal elements [7] and/or a sterol carrier protein [8], to the outer membrane of mitochondria. This process is probably not needed for the acute response, but is necessary for sustained synthesis of steroid hormones. Cholesterol is converted to pregnenolone in the inner mitochondrial membrane [9,10] by the cholesterol side-chain cleavage complex, which consists of three proteins: a flavoprotein, NADPH:adrenodoxin oxidoreductase; a 2Fe–2S* ferredoxin, adrenodoxin; and a hemoprotein, cytochrome $P450_{scc}$. The cytochrome is reduced by the adrenodoxin and is the site of O_2 and steroid substrate binding as well as catalysis. These proteins are not synthesized as part of the acute response to ACTH, nor is there any evidence that any of them is phosphorylated *in vivo* as part of the stimulatory process. The cholesterol side-chain cleavage reaction is the initial and rate-limiting step [11,12] in the steroid hormone biosynthetic pathway. It is probable that the cause of this limitation is substrate availability and that the transport of cholesterol from the outer to the inner mitochondrial membrane is the actual rate-limiting process [13].

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This conclusion is based in part on the observation that even in cells stimulated maximally, either *in vivo* or *in vitro*, the enzyme is not functioning at its full catalytic capacity.

Another phosphoprotein, which is produced [14] in response to ACTH or cAMP stimulation of adrenal cells, is pp28 (formerly i_b). pp28 has been shown to be phosphorylated [15] on serine or threonine (L. A. Pon and N. R. Orme-Johnson, unpublished results). The unphosphorylated form p28 (formerly p_b) is synthesized in unstimulated cells but is not converted to pp28 in response to ACTH or cAMP action. This is probably because p28 is transported into the mitochondria [16] and is thus not accessible as a substrate for a cytosolic kinase. The synthesis of pp28, quantified by visualization of two-dimensional gels, has been extensively correlated with the increased rate of synthesis of steroid hormones in several steroidogenic tissues from a variety of species [14–18]. Thus, the conditions for its synthesis and the subcellular localization of this mitochondrial protein, as well as the fact that it is phosphorylated, are consistent with its serving a function in the "acute" response of steroid-producing tissues.

This "acute" response has also been shown to be greatly affected by protein synthesis inhibitors [19–22]. The administration of cycloheximide or puromycin prior to ACTH or cAMP *in vivo* or *in vitro* prevents the increase in the rate of steroidogenesis. Additionally, the administration of such protein synthesis inhibitors after ACTH or cAMP causes a rapid decline in the rate of synthesis of glucocorticoids. It was this observation, in combination with the results of experiments utilizing inhibitors of RNA synthesis and of the enzymes in the steroidogenic pathway, that allowed Garren *et al.* [1] to propose that a short-lived, but newly-synthesized protein may be involved in "acute" steroidogenesis. The current theory is that inhibitors of protein synthesis are blocking ACTH-stimulated steroid production by preventing the synthesis of a protein required for the transport of cholesterol from the outer to the inner mitochondrial membrane [13], thus preventing the interaction of cholesterol with cytochrome $P450_{scc}$. This process is thought to be mediated by the protein proposed by Garren *et al.* [1]. In fact, the synthesis of pp28 in response ACTH or cAMP is inhibited by cycloheximide, indicating that only newly-synthesized p28 may be phosphorylated to produce pp28. Thus, pp28 is a possible candidate for Garren *et al.*'s newly-synthesized protein.

Although protein sythesis inhibitors have been crucial to our understanding of the regulation of steroidogenesis in the cell, the action of these compounds is not completely characterized and is almost certainly not confined to the inhibition of protein synthesis. Certain of these inhibitors can be quite cytotoxic. The use of amino acid analogues is an alternative method of studying the mechanisms of regulation of steroid hormone production [23,24]. These analogues are structurally very similar to the 20 amino acids normally found in mammalian proteins. The specific aminoacyl tRNA synthetases can recognize these analogues and can incorporate them in place of the natural amino acids, resulting in the formation of altered proteins. The rationale for employing these analogues is to "mutate" key residues in the newly-synthesized proteins within the cell, hopefully impairing the function of such proteins, without inhibiting overall protein synthesis. In this way, amino acid analogues are much more specific than protein synthesis inhibitors, since analogues of suspected key residues can be used to try to produce more specific interference. Additionally, concentrations of analogues can be chosen which are not cytotoxic to the cell. This technique has been used to investigate the processing of preproteins [25–27] and prohormones [28–31] in whole cells and tissues, as well to investigate asparagine-linked glycosylation [32, 33] in cell-free systems. Amino acid analogues have been shown to provide more detailed information about important functional amino acids of the protein(s) involved in the individual process under investigation. This approach may yield new insights into the involvement of a newly-synthesized protein in "acute" steroidogenesis.

In previous studies from this laboratory [34], amino acid analogues of most of the 20 amino acids found in mammalian proteins have been incubated with isolated rat adrenal cortex cells and the effects of each analogue on overall protein synthesis and on steroidogenesis have been investigated. Two of these analogues, L-canavanine (an arginine analogue) and L-*S*-aminoethylcysteine (an analogue of lysine), were found to inhibit corticosterone synthesis to a much greater extent than they inhibited protein synthesis. The observed effects occurred between the formation of cAMP the production of pregnenolone and could be reversed by the

addition of the natural amino acid. These studies provide additional support for a protein involved in "acute" steroidogenesis and suggest that lysine, and possibly arginine, may be important amino acids in the function of this protein.

In this study, the effect of serine and theonine analogues on the production of corticosterone in rat adrenal cortex cells has been investigated. We wished to explore whether proteins synthesized after ACTH stimulation, such as pp28, would incorporate these analogues, and be nonfunctional due to their inability to be phosphorylated by protein kinase A. A specific analogue of threonine, D,L-β-hydroxynorvaline, was found to inhibit steroidogenesis, almost completely, at micromolar concentrations by a mechanism seemingly independent of protein synthesis inhibition. This effect was totally reversed when equal molar concentrations of threonine were present along with the analogue. β-Hydroxynorvaline also prevented the incorporation of radiolabelled threonine into protein. These experiments suggest that β-hydroxynorvaline is indeed functioning as an analogue of threonine and that threonine phosphorylation may be important in the regulation of "acute" steroidogenesis.

EXPERIMENTAL

Materials

Female Sprague–Dawley rats (151–200 g) were purchased from Taconic Farms. Porcine ACTH, dibutyryladenosine-3′:5′-cyclicmonophosphate (Bt_2cAMP), collagenase (type I), deoxyribonuclease (DNase), ribonuclease (RNase), D,L - β - hydroxynorvaline, D,L - isoserine, α - methyl-D,L-serine, L-homoserine and L-threonine were obtained from Sigma. Pregnenolone was from Steraloids Inc. [^{3}H]Corticosterone and [^{3}H]L-threonine were purchased from Amersham Corp.; cell-labelling grade [^{35}S]methionine was from Du Pont/New England Nuclear. Antisera for corticosterone radioimmunoassay (RIA) were obtained from ICN. Other reagents were of the highest quality commercially available.

Preparation of rat adrenal cortex cells

Adrenal cells were isolated according to the procedure of Ray and Strott [35] with some minor modifications described below. Rats were anaesthetized using CO_2, followed by decapitation. The adrenal glands from 12 rats were excised, decapsulated and placed on ice in a Krebs–Ringer bicarbonate buffer [36] containing 0.5% bovine serum albumin and 0.2% glucose (KRBAG). The tissue was minced and digested in 6 ml of KRBAG containing collagenase (2 mg/ml) and DNase (0.5 mg/ml) for 30 min at room temperature, followed by 20 min at 37°C. Digested tissue fragments were allowed to settle to the bottom of the tube (15 ml conical, Falcon) and the collagenase/DNase solution was discarded. The tissue was resuspended in 6 ml of KRBAG and the cells were released by triturating the suspension using a disposable 10 ml pipet. Isolated cells were collected through centrifugation (100 *g*, 10 min, 30°C) and washed 3 times in KRBAG. Remaining tissue pieces were subjected to a second digestion and trituration. Washed cells were resuspended in 6 ml KRBAG and incubated for 1 h at 37°C to insure that the cells were synthesizing a low, unstimulated amount of corticosterone. After this incubation, the cells were washed 3 additional times in KRBAG. Cell viability was determined by erythrosin B exclusion [14] and the cells were resuspended at 500,000 cells/ml in KRBAG.

Corticosterone synthesis

To measure steroid hormone production in response to stimulant, rat adrenal cortex cells were aliquoted into 12 × 75 mm polypropylene tubes (Falcon 2054) to achieve a final cell concentration of 40,000 cells/ml in KRBAG. Cells were incubated at 37°C in a shaking water bath. Final volume of each incubation was 1.8 ml. ACTH (1 μg/ml),Bt_2cAMP (2 mM) or pregnenolone (5 μM) was added; 500 μl of the cell suspension was removed at 0, 10 and 20 min after stimulation and quenched into 500 μl of ice-cold water in a 12 × 75 mm disposable glass tube. Stimulant was not added to control samples. Appropriate amounts of β-hydroxynorvaline and/or threonine were added 30 min prior to stimulant addition. Corticosterone was measured by RIA. Rates of corticosterone production were determined by plotting the amount of corticosterone synthesized (ng/ml) vs time (min). Least-squares analysis was used to determine the best fit through the data points and the slope of the line/number of cells/ml in the incubation produced a rate of corticosterone synthesis expressed as ng/cell/min. The ACTH rate of corticosterone production for each cell preparation is defined as 100%.

Protein synthesis measurements

To determine overall protein synthesis, rat adrenal cortex cells were aliquoted into 1.5 ml Eppendorf tubes to a final concentration of 40,000 cells/ml in KRBAG. Cells were incubated at 37°C in a shaking water bath. The final volume of the incubation was 0.8 ml. 5 Min after the addition of ACTH (1 μg/ml), [^{35}S]methionine (20 μCi/ml) or [^{3}H]threonine (10 μCi/ml) was added. 200 μl Aliquots were removed at 0, 10 and 20 min after label addition and quenched into 250 μl of ice-cold water in Eppendorf tubes. Control samples did not contain ACTH. Appropriate amounts of β-hydroxynorvaline were added 30 min prior to ACTH addition.

The amount of radioactivity incorporated into protein was determined by trichloroacetic acid (TCA) precipitation of solubilized samples by a modification, described below, of the procedure of Laemmli [34]. Each time point was treated with 20 μl of DNase (0.5 mg/ml) RNase (0.25 mg/ml) and 20 μl of Laemmli SDS-sample buffer, vortexed and heated to 90°C for 5 min; 0.5 ml of 20% TCA was added to each sample and the samples were placed in a 90°C water bath for 20 min. Samples were cooled to 0°C in ice-water for at least 1 h. The precipitated material was collected on GF/A filters (Whatman). The filters were washed extensively with 5% TCA, rinsed with 95% ethanol, air-dried, treated with nonaqueous liquid scintillation fluid and counted in a Beckman LS 3801 scintillation counter. Rates of radiolabel incorporation were obtained by plotting cpm vs time for each incubation. Least-squares analysis was used to determine the best line through the data points. ACTH rate of radiolabel incorporation for each cell preparation is defined as 100%.

RESULTS

The effect of analogues of serine and threonine on steroidogenesis

Since pp28 has been shown to be phosphorylated on serine or threonine, analogues of serine and threonine were used in an attempt to inhibit steroidogenesis. The rationale was to inhibit or perturb phosphorylation of newly-synthesized p28 by displacing the natural amino acids with the analogues. This experiment would test the hypothesis that phosphorylation of p28 or some other protein, synthesized after ACTH stimulation, is necessary for steroidogenesis. Table 1 lists analogues of serine and threonine that were tested for their effect on steroid hormone production. All analogues of serine (D,L-isoserine, α-methyl-D,L-serine and L-homoserine) showed little to no effect on ACTH-stimulated corticosterone production in isolated rat adrenal cortex cells at analogue concentrations of 5 mM as compared with steroid hormone production in cells exposed to ACTH alone. However, the highly-specific threonine analogue, β-hydroxynorvaline, almost completely inhibited ACTH-stimulated steroidogenesis at 5 mM. This dramatic inhibition had not been observed in previous studies when a number of other amino acid analogues of the 20 natural amino acids found in proteins had been tested for their effect on steroidogenesis.

Table 1. The effect of amino acid analogues of serine and threonine on corticosterone production by isolated rat adrenal cortex cells

Amino acid	Analogue	Corticosterone production (% ACTH)[a]
Serine	D,L-Isoserine	82
	α-Methyl-D,L-serine	96
	L-Homoserine	94
Threonine	D,L-β-Hydroxynorvaline	4

Suspensions of rat adrenal cortex cells (40,000 cells/ml) were preincubated with 5 mM of the analogue for 30 min at 37°C. Porcine ACTH (1 μg/ml) was added and the cell suspensions were incubated for an additional 30 min and assayed for corticosterone production by RIA.

[a] The amount of corticosterone produced by ACTH-stimulated cells in the absence of analogues was 1.30×10^{-5} ng/cell/min. There was virtually no corticosterone production in the absence of ACTH.

To determine the concentration dependence of steroid hormone inhibition by β-hydroxynorvaline, rat adrenal cortex cells were incubated with varying concentrations of analogue 30 min prior to stimulation by ACTH. Aliquots of the reaction mixture were removed upon addition of ACTH, as well as 10 and 20 min after stimulation and quenched into ice-cold water to stop steroid hormone production. The individual samples were analysed for corticosterone. Figure 1(a) illustrates the dose-dependency of corticosterone inhibition by β-hydroxynorvaline. At approx. 30 μM, corticosterone production is inhibited by 50%; 300 μM β-hydroxynorvaline inhibits steroid hormone synthesis to the same extent, i.e. *ca* 95%, as 5 mM.

The effect of β-hydroxynorvaline on protein synthesis

To determine whether the inhibition of steroidogenesis by β-hydroxynorvaline is due to a concomitant inhibition of protein synthesis, a profile of overall protein synthesis in the

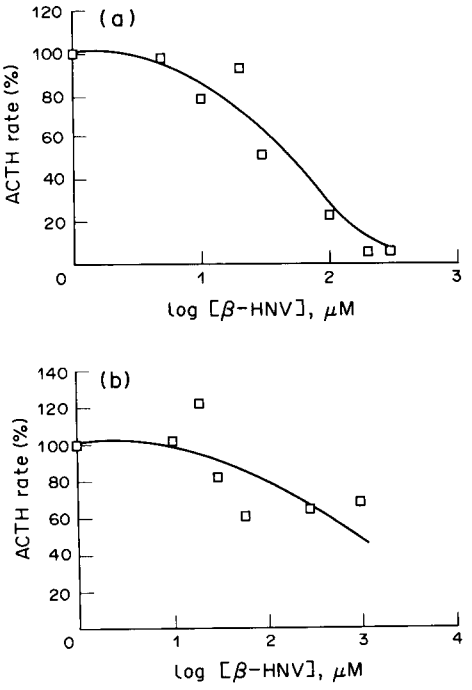

Fig. 1. The inhibition of steroidogenesis and protein synthesis by β-hydroxynorvaline. (a) Rat adrenal cortex cells were incubated for 30 min with the concentrations of β-hydroxynorvaline indicated. ACTH (1 μg/ml) was added and aliquots of the cell suspension were removed and quenched into ice-cold water at 0, 10 and 20 min after stimulation. The amount of corticosterone produced was determined by RIA. The rate of corticosterone synthesis was calculated by plotting the amount of steroid hormone (ng/ml) vs time and fitting the data by least-squares analysis. The rates were normalized to the rate obtained with ACTH and no analogue. The numbers shown are the means of data obtained from 1–5 cell preparations with duplicates performed in each experiment. The ACTH-stimulated rate of corticosterone synthesis was 5.98×10^{-5} ng/cell/min. (b) Incubation of adrenal cortex cells was performed and samples were removed as described for the experiments shown in part (a), except that ACTH was added 5 min prior to the addition of [^{35}S]methionine (20 μCi/ml) at $t = 0$. The incorporation of radiolabelled amino acid into protein in each sample was determined by TCA precipitation of solubilized samples, followed by scintillation counting of this material. Calculation of the rate of radiolabel incorporation and normalization were carried out as for part (a). The data represent measurements from 1–3 cell preparations with duplicates performed in each experiment.

presence of varying concentrations of analogue was determined. Rat adrenal cortex cells were incubated with varying concentrations of analogue for 30 min prior to stimulation by ACTH. A small amount of [^{35}S]methionine was added 5 min after ACTH addition and aliquots of the reaction were removed immediately after the addition of label, as well as 10 and 20 min after label addition. The aliquots were quenched into ice-water, solubilized and precipitated with TCA. Insoluble precipitated material was collected on glass filters, which were subsequently treated with scintillation fluid and counted in a scintillation counter. Rates of radiolabel incorporation were determined for several concentrations of β-hydroxynorvaline and the rates were normalized to that obtained for ACTH. Figure 1(b) demonstrates that protein synthesis is inhibited to some extent upon the addition of β-hydroxynorvaline, however the inhibition is much less than that seen for steroidogenesis. Thus, at 200–300 μM β-hydroxynorvaline, 50–60% of the steroidogenesis inhibition is a result of a mechanism other than that of the inhibition of protein synthesis. A similar profile was determined in the presence of analogue but in the absence of ACTH (data not shown). The profile was identical to that obtained when stimulant is added. Subsequent studies will use the concentration of 300 μM β-hydroxynorvaline in order to characterize the above observations to a greater extent.

β-Hydroxynorvaline is a specific analogue of L-threonine

In order to determine that β-hydroxynorvaline is indeed acting as an amino acid analogue of threonine, corticosterone measurements were made in the presence of analogue (300 μM) and varying concentrations of threonine (Fig. 2). Both analogue and threonine were added at the same time, which was 30 min prior to ACTH stimulation. At 3 μM threonine, reversal of the inhibition of β-hydroxynorvaline is observed; by 300 μM threonine, the ACTH-stimulated rate of corticosterone production is restored.

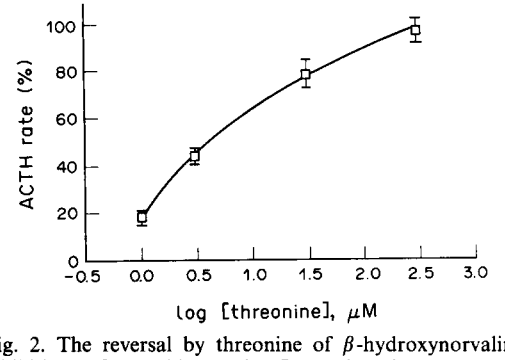

Fig. 2. The reversal by threonine of β-hydroxynorvaline inhibition of steroidogenesis. Rat adrenal cortex cells (40,000 cells/ml) were incubated with 300 μM β-hydroxynorvaline and varying concentrations of threonine for 30 min. The cells then were stimulated with ACTH. Samples were removed at 0, 10 and 20 min and quenched into ice-cold water, followed by corticosterone determination by RIA. Rates were determined as described for Fig. 1. The data represents rates for 2–4 cell preparations with duplicates performed for each experiment. The rate of corticosterone synthesis in samples exposed to ACTH alone was 8.80×10^{-5} ng/cell/min.

In another experiment, the incorporation of [^{3}H]threonine in the presence and absence of 300 μM β-hydroxynorvaline was determined. The analogue was introduced into adrenal cortex cells either 30 min before ACTH addition or along with ACTH. [^{3}H]Threonine was added 5 min after ACTH and aliquots were removed immediately after label addition, as well as 10 and 20 min later. Radiolabel incorporation was determined as described previously. Figure 3 shows that threonine incorporation into proteins is severely inhibited in the presence of analogue, only 17% of the incorporation seen with ACTH alone. This observation is true whether or not the cells were preincubated with the analogue. This experiment was also performed under unstimulated conditions, producing the same results seen in the stimulated case (data not shown). These two experiments suggest that β-hydroxynorvaline is functioning as an amino acid analogue in adrenal cortex cells.

Locus of steroid hormone inhibition

The experiment stated above suggests that β-hydroxynorvaline is inhibiting steroidogenesis by being incorporated into the newly-synthesized protein in place of threonine, thus producing non-functional proteins; in this case, ones that may not be phosphorylated. However, to examine the possibility that this analogue may be inhibiting other reactions in the steroidogenic or the ACTH-stimulated signal transduction pathways, the following experiments were performed. Rat adrenal cortex cells, preincubated with 300 μM β-hydroxynorvaline for 30 min were stimulated with a soluble analogue of cAMP, Bt_2cAMP, and the rate of corticosterone production was measured. Table 2 illustrates that the ACTH-stimulated rate of steroid hormone production was achieved when the cells were stimulated with Bt_2cAMP. To check for inhibition of enzymes in the steroidogenic pathway, pregnenolone was added to rat adrenal cortex cells and the rate of corticosterone synthesis was measured. The 3 to 5-fold increase in the rate of corticosterone synthesis observed suggests that the steroidogenic pathway subsequent to the conversion of cholesterol to pregnenolone is not inhibited by the analogue.

Table 2. β-Hydroxynorvaline inhibition of steroidogenesis occurs between the production of cAMP and the synthesis of pregnenolone

Corticosterone production	(% ACTH rate)[a]
– β-Hydroxynorvaline	
ACTH	100
cAMP	115 ± 8
Pregnenolone	330 ± 65
+ β-hydroxynorvaline	
ACTH	17 ± 7
cAMP	28 ± 5
Pregnenolone	488 ± 152

[a]The amount of corticosterone produced by ACTH-stimulated cells in the absence of analogues was 1.16×10^{-4} ng/cell/min. There was virtually no corticosterone synthesized in the absence of stimulant. The data represent values from 2–3 cell preparations, duplicates performed for each cell preparation.

Steroidogenesis is inhibited immediately upon addition of β-hydroxynorvaline

The experiments described above allowed the cells to be incubated with the analogue for

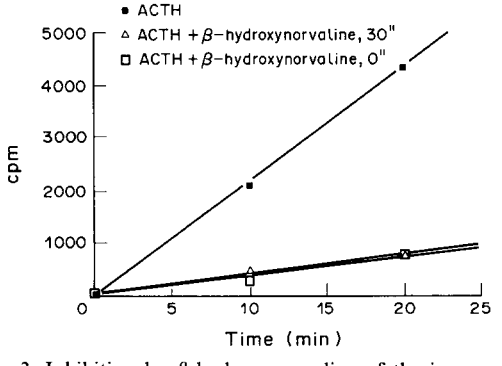

Fig. 3. Inhibition by β-hydroxynorvaline of the incorporation of threonine into newly-synthesized proteins. Rat adrenal cortex cells (40,000 cells/ml) were stimulated with ACTH, followed by the addition of [^{3}H]threonine (10 μCi/ml) 5 min later (■). 300 μM β-Hydroxynorvaline was added 30 min prior to ACTH addition (△) or at the same time as ACTH (□). Aliquots of cells were removed 0, 10 and 20 min after label addition and quenched into ice-cold water. Determination of the amount of radiolabelled amino acid incorporated into protein and calculation of the rates of this process for the different samples were carried as described for Fig. 1.

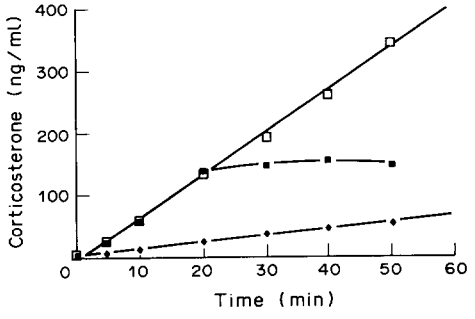

Fig. 4. Time course of inhibition of steroidogenesis by β-hydroxynorvaline. Rat adrenal cortex cells (40,000 cells/ml) were simulated with ACTH and 300 μM β-hydroxynorvaline was added 20 min after this stimulant (■). In another sample, both ACTH and 300 μM β-hydroxynorvaline were added at the same time (◆). In a third sample the cells were treated with ACTH alone (□). Aliquots were removed at various times, quenched into ice-cold water, assayed for corticosterone and the rates calculated as for Fig. 1.

30 min before stimulation. This was to insure that the analogue was equilibrated with the amino acid pools used in translation. However, as Fig. 4 illustrates, the effect of 300 μM β-hydroxynorvaline on corticosterone production is immediate. When the analogue is added 20 min after adrenal cortex cells are stimulated with ACTH, corticosterone synthesis is inhibited and the amount of corticosterone in the incubation mixture remains essentially constant up to 50 min. If ACTH and β-hydroxynorvaline are added at the same time, the response to ACTH is prevented and a low level of corticosterone is produced throughout this 50 min window.

DISCUSSION

In previous studies, a number of amino acid analogues have been incubated with isolated rat adrenal cortex cells and the inhibition of protein and steroid synthesis investigated [34]. Most of these compounds were either ineffective at low, non-cytotoxic concentrations or else inhibited protein and steroid hormone synthesis to the same extent. However, two of the analogues were found to inhibit corticosterone synthesis to a much greater extent than they inhibited overall protein synthesis. Canavanine, an analogue of arginine, inhibited steroid hormone synthesis by approx. 60% at 5 mM; *S*-aminoethylcysteine, a lysine analogue, blocked corticosterone production by approx. 75% at 5 mM. In both cases protein synthesis was inhibited by only approx. 25%. The onset of inhibition was rapid and the inhibition could be prevented by adding an equal concentration of the natural amino acid concurrent with analogue addition. It was also determined that these analogues were inhibiting the steroidogenic pathway between the formation of cAMP and the production of pregnenolone. In addition, tritiated *S*-aminoethylcysteine was shown to be incorporated into proteins and the presence of *S*-aminoethylcysteine inhibited the incorporation of tritiated lysine into protein. These studies provide additional support for a newly-synthesized protein involved in "acute" steroidogenesis and it appears that lysine, and possibly arginine, may be important amino acids in the function of this protein. The importance of lysine is especially interesting in light of the experiments of Kido and Kumura [38], in which low concentrations of poly-L-lysine were shown to stimulate the binding of liposomal cholesterol to steroid-free cytochrome $P450_{scc}$. In addition, mitochondrial targeting pre-sequences are basic in nature, due to the presence of lysine and arginine [39]. Since pp28 has been shown to be a mitochondrial protein, the inhibition of steroidogenesis may be partially due to an ability of the protein containing the analogues to be imported into the mitochondrion. This inability to become a mitochondrial protein would leave pp28 stranded in the cytosol, thus preventing it from functioning as a facilitator in the interaction of cholesterol with cytochrome $P450_{scc}$.

In this study, we have continued our investigations of the effect of amino acid analogues on steroidogenesis. This was done, both in order to check further the hypothesis that a newly-synthesized protein is involved in ACTH-mediated steroid hormone production and to try to establish that this protein is a phosphorprotein. Thus, analogues of serine and threonine were used to explore the role of phosphorylation in steroidogenesis. The three analogues of serine (isoserine, α-methylserine, homoserine) had little to no effect on stimulated steroidogenesis. However, the threonine analogue, β-hydroxynorvaline, inhibited corticosterone production almost completely (*ca* 95%) at concentrations as low as 300 μM. Since this concentration of analogue inhibits protein synthesis by *ca* 40%, part of the decrease in steroidogenesis may be attributable to this inhibition of protein synthesis. However, another mechanism must be postulated to explain the additional inhibition of steroidogenesis observed in rat adrenal cortex cells.

Evidence to suggest that β-hydroxynorvaline is acting as an analogue, i.e. replacing threonine in proteins synthesized after addition of the analogue, are the following. The inhibition of steroidogenesis occurs rapidly after the addition of analogue, which suggests that it is being incorporated readily into newly-synthesized proteins. The inhibition can be completely reversed when an equal molar concentration of threonine is present. Further, β-hydroxynorvaline prevents the incorporation of [^{3}H]threonine into proteins. It is particularly interesting to note that the percentage inhibition of radiolabelled threonine into proteins by the analogue parallels very closely the percentage inhibition of steroidogenesis. Thus, while inhibition of overall protein synthesis may account for some of the inhibition of steroidogenesis, the bulk of this decrease in steroidogenesis may be due to the replacement of threonine by β-hydroxynorvaline. Thus, if phosphorylation of a newly-synthesized protein is essential for the acute

increase in the rate of steroidogenesis produced by ACTH and if the protein needed contains β-hydroxynorvaline, the protein may be made "non-functional" due to its inability to be phosphorylated by protein kinase A.

This assertion is consistent with the observation that the analogue is inhibiting the cellular processes subsequent to the produon of cAMP and prior to the synthesis of pregnenolone. β-Hydroxynorvaline has no effect on ACTH binding to its receptor or the stimulation of adenylate cyclase as evidenced by the fact that the rates of corticosterone synthesis in the presence of a soluble form of cAMP, Bt_2cAMP, in the presence or absence of the analogue, parallels the values obtained when ACTH is used as the stimulant. In addition, the presence of pregnenolone results in the characteristic 3 to 5-fold increase in the rate of corticosterone synthesis and is unchanged by the presence of the analogue. The newly-synthesized protein, proposed by Garren *et al.* [1] on the basis of studies using protein synthesis inhibitors, is thought to function between the production of cAMP and pregnenolone, i.e. the same region in which β-hydroxynorvaline exerts its effect. Thus, there is evidence to suggest that this analogue is affecting a protein involved in the "acute" phase of steroidogenesis. Whether or not this protein is a phosphoprotein is being investigated currently.

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