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THE ROLE OF LYSINE-RICH PROTEINS IN THE ACUTE STEROIDOGENIC RESPONSE OF RAT ADRENAL CELLS TO ACTH

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SUMMARY: The possibility that a lysine-rich protein was involved in the acute stimulation of steroidogenesis by ACTH was investigated using [^{3}H] labelled lysine and isolated adrenal cells. The results demonstrated that cycloheximide inhibited steroidogenesis in a dose-dependent, rapid fashion and inhibited the incorporation of radioactive lysine into protein. However cells incubated in a lysine-free medium showed the same response to ACTH as cells incubated in a lysine-containing medium. It was also demonstrated that ACTH had no effect on the incorporation of tritiated lysine into the protein or small peptide fractions. These observations suggest that a rapidly synthesised, lysine-rich protein is not involved in the acute response to ACTH.

INTRODUCTION : ACTH stimulates steroidogenesis in the adrenal cortex by increasing the rate of conversion of cholesterol to pregnenolone (1,2), the rate limiting step in steroid hormone synthesis (1-3). Cycloheximide inhibits ACTH stimulation of steroidogenesis (4,5), however the exact mechanism of this inhibition is not known. It has been suggested that cycloheximide inhibits the synthesis of a rapidly synthesised, labile, protein factor that is involved in the transport of cholesterol to the side-chain cleavage enzyme (6-8) and that ACTH stimulates the synthesis of this protein hence increasing the amount of substrate available for pregnenolone production. Little information is available on the possible properties of this factor, however recent observations have suggested that it may be a lysine-rich protein (9,10). Tthe purpose of the study described below was to test this hypothesis by investigating the effect of ACTH on the incorporation of radioactive lysine into protein by isolated adrenal cells.

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MATERIALS and METHODS: Cycloheximide was obtained from Koch-Light Ltd., Colnbrook, Bucks., U.K. Lysine-free Dulbecco's Modified Eagles medium was from Gibco Ltd., Paisley,U.K. and Sephadex G-10 from Pharmacia, Hounslow, Middlesex, U.K. L- [4,5-^{3}H] Lysine was purchased from the Radiochemical Center, Amersham, Bucks., U.K. Details of other materials given in (11).
Cells, isolated as described in (11), were preincubated in 500µl of lysine-free Dulbecco's Modified Eagles medium containing 5 µCi of ^{3}H lysine for 20 min at 37°C, then incubated with appropriate additions for 1h at 37°C. At the end of the incubation 1 ml of 0.6M NaOH and 0.2 ml of 30% hydrogen peroxide was added and this solution was incubated at 37°C for 10 min. 1 ml of 20% trichloroacetic acid (TCA) was added and the precipitated protein was collected by centrifugation at 5000xg for 2 min at 2°C. The precipitate was washed three times by resuspension in 1N NaOH and precipitation in 20% TCA. Finally the precipitate was resuspended in 250 µl of 1N NaOH, neutralised to a final volume of 500µl and a 100µl sample taken for counting in a Packard LS 3000 scintillation counter.
The incorporation of ^{3}H lysine into small peptides (i. e. mol. wt. $>$700 but too small to be precipitated by TCA) was assayed by applying the neutralised TCA soluble fraction on to a Sephadex G-10 column. The column was eluted with phosphate-buffered saline (pH 7.4). 60 x 1.5 ml fractions were collected from the column and 500 µl samples were counted. Neutralised, resuspended TCA precipitates were chromatographed in the same way.
The method used for cell incubation and radioimmunoassay of corticosterone has been described previously(11).

RESULTS and DISCUSSION: Figure 1a shows the dose-response characteristics of cycloheximide inhibition of ACTH-stimulated steroidogenesis. As has been shown previously (12), cycloheximide is a potent inhibitor with the ED_{50} for inhibition at 1 µM and greater than 90% inhibition at 10 µM cycloheximide. Note that in future experiments a cycloheximide concentration of 20 µM was used. Figure 1b shows that the effect of cycloheximide was very rapid, in agreement with previous observations (4,12), and suggests that a protein with a half-life of approximately 5 min in ACTH action.

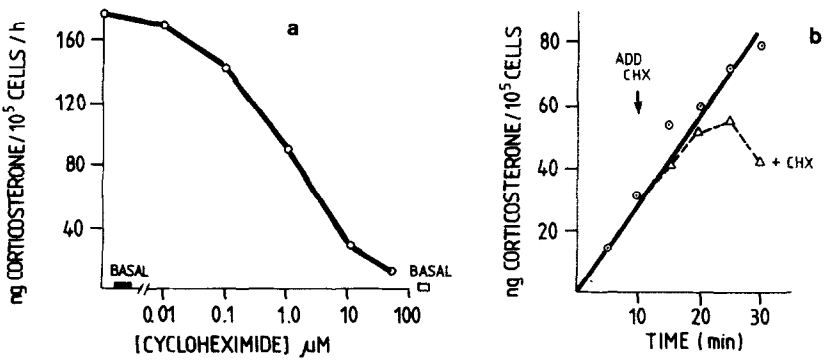

FIGURE 1: The effect of cycloheximide on adrenal cell response to ACTH : Isolated rat adrenal cells were incubated with varying concentrations of cycloheximide for 1h (1a), or with 20 µM cycloheximide for varying times (1b). At the end of each incubation corticosterone was extracted and assayed as in (11). ACTH was added as 10 mU.

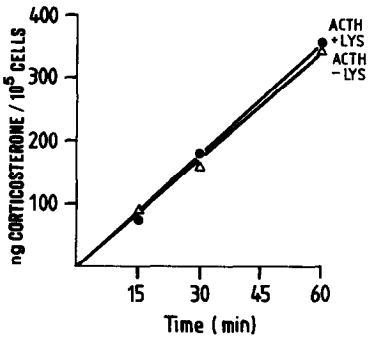

FIGURE 2 : The effect of lysine deprivation on adrenal cell response to ACTH: Isolated rat adrenal cells were incubated in the presence of 10 mU of ACTH and in the presence (●——●) or absence (Δ——Δ) of lysine (73 µg/ml).Corticosterone was assayed by radioimmunoassay (11).There was no detectable steroid synthesis in the absence of ACTH.

The role of lysine in the synthesis of this protein (9,10) was investigated. The ommission of lysine from the incubation medium has no effect on the ability of the cells to respond to ACTH (Figure 2) suggesting there is no chronic requirement for lysine in ACTH action. However the possibility remains that the cells are using lysine obtained from the degradation of proteins added to the medium i.e. bovine serum albumin or ACTH, or by the recirculation of lysine derived from the degradation of intracellular protein. Table 1 also suggests that the ACTH sensitive protein is not lysine-rich, it shows that ACTH did not stimulate ^{3}H lysine incorporation into TCA precipitable material while stimulating steroidogenesis, however cycloheximide inhibits both steroid synthesis (90%) and lysine incorporation (45%). The possibility that ACTH may stimulate the incorporation of lysine into an active peptide which is

TABLE 1

The effect of ACTH and cycloheximide on the incorporation of ^{3}H lysine into TCA precipitable and on steroidogenesis

Addition	c.p.m. x 10^{-4}/ 10^5 cells/h	ng steroid/ 10^5 cells/h
None	9.95	20.7
ACTH (10 mU)	9.75	261.7
ACTH (10 mU) +Cycloheximide (20 µM)	5.53	28.0

Cells were isolated and incubated and TCA precipitable material prepared as described in "Materials and Methods". Corticosterone was assayed by radioimmunoassay (11).

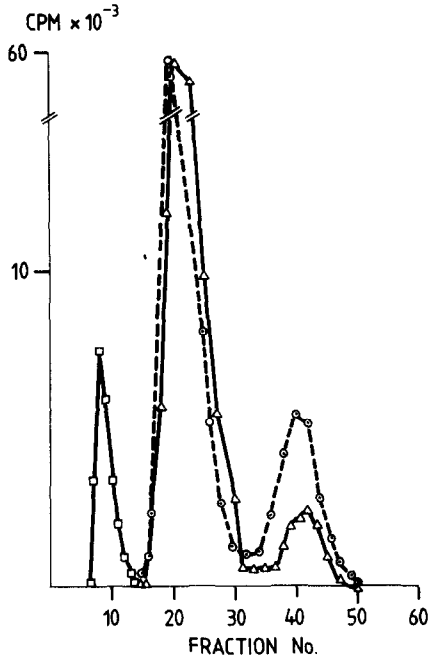

FIGURE 3: Sephadex G-10 chromatography of TCA soluble and insoluble fractions and ^{3}H lysine: Samples were prepared, chromatographed and counted as described in "Materials and Methods" The samples shown are: (□—□) TCA-precipitable material : (△——△) TCA-soluble material : (⊙——⊙) ^{3}H lysine

too small to be precipitated by TCA was investigated. The TCA soluble material was chromatographed on Sephadex G-10. Figure 3 shows that there was no radioactive material of molecular weight >700daltons synthesised which was not precipitated by TCA, whether ACTH was present or not, suggesting that ACTH does not stimulate the synthesis of lysine-rich peptides or proteins. It must be emphasised however, since nothing is known about the size of the lysine pool available for protein synthesis, or the ability of added lysine to penetrate this pool, nor is there anything known about the degradation of the cycloheximide sensitive factor, thus any results obtained using added radioactive amino acid are very difficult to interpret (13). In support of the conclusion that the ACTH dependent protein is not lysine-rich is a recent report by Pederson & Brownie (14), which describes an ACTH, cycloheximide sensitive peptide which can stimulate cholesterol side-chain cleavage, this peptide is not rich in lysine residues.

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