ACTH-DEPENDENT STIMULATION OF A SPECIFIC PEPTIDE IN ADRENOCORTICAL CELLS IN CULTURE

A. Dazord, D. Gallet and J.M. Saez

Unité de Recherches sur le Contrôle Hormonal des Activités Cellulaires (INSERM, U. 162). 29 Rue Soeur Bouvier. 69322 LYON Cedex 1. France

Received May 12,1977

SUMMARY

Corticotropin (1-24) tetracosapeptide ($ACTH_{1-24}$) induces a small but significant increase in the incorporation of radioactive leucine into trichloracetic insoluble proteins of a mouse adrenal cell line Y_1. Neither cyclic AMP, nor cholera toxin or a nitrophenyl sulfenyl derivative of $ACTH_{1-24}$ (NPS-$ACTH_{1-24}$) have any effects.

After being labelled with radioactive leucine in the presence or absence of ACTH, the cells were solubilized in 1 % sodium dodecylsulfate and subjected to 20 % sodium dodecylsulfate polyacrylamide gels electrophoresis. $ACTH_{1-24}$ was found to induce a dramatic increase in the incorporation of radioactive leucine into a small peptide (MW 3500). This effect was mimicked by other steroidogenic compounds such as cholera toxin, cyclic AMP, NPS-$ACTH_{1-24}$ but not by $ACTH_{11-24}$, a non steroidogenic analogue of ACTH.

INTRODUCTION

Data concerning ACTH's effects on adrenal protein synthesis are complex and contradictory. It is well established that protein synthesis is an obligatory step of the ACTH stimulated steroidogenesis, since inhibitors of protein synthesis block the action of ACTH (1). In vivo trophic effects of ACTH are well known (2). In vitro results are controversial. Some authors have found that ACTH treatment inhibits total protein synthesis (3-5) but others disagree (6, 7). On the other hand, it is well established that ACTH can selectively increase specific mitochondrial enzymes (8, 9) or some cytosolic proteins (10). But in both cases it has been shown that the specific increase of these proteins cannot account for a possible stimulatory effect of ACTH on total adrenal protein synthesis or even on total mitochondrial protein synthesis.

The data of the present work indicate that among all the steroidogenic substances tested ($ACTH_{1-24}$, its o-nitrophenyl sulfenyl derivative NPS-$ACTH_{1-24}$,

ACTH = adrenocorticotropic hormone. DbcAMP = dibutyryl cyclic adenosine 3'5'-monophosphate. $ACTH_{1-24}$ = Corticotropin (1-24) tetracosapeptide. NPS-$ACTH_{1-24}$ = (9-tryptophan(o-nitrophenyl sulfenyl))-corticotropin-(1-24)-tetracosapeptide. 20αOH progesterone = 20α-hydroxypregn-4-ene-3-one.

ISSN 0006-291X

DbcAMP, and cholera toxin), only $ACTH_{1-24}$ was able to increase total protein synthesis, but all of them induce a dramatic increase (50 to 100 %) of a small peptide.

MATERIAL AND METHODS

$ACTH_{1-24}$ was provided by CIBA. NPS-$ACTH_{1-24}$ and $ACTH_{11-24}$ were a gift from Drs. Rittel and Desaulles (Ciba-Geigy AG, Basel, Switzerland). Cholera toxin was a gift from Dr. C.E. Miller (NIH, Bethesda). [^{3}H]-leucine (specific activity, 25 to 35 Ci/mmole) and [^{14}C]-leucine (specific activity, 40 to 55 mCi/mmole) were obtained from Saclay, France.

Functional cloned mouse adrenal tumor cells (Y_1) developped in the laboratory of G. Sato (10) were purchased from the American Culture Cell Repository (Rockville, Md). They were grown in Ham's F-10 medium supplemented with 10 % heat-inactivated horse serum and 2.5 % heat-inactivated fetal calf serum (FCS).

The incorporation of [^{14}C]-leucine in proteins was measured in the cells 2 days after seeding in petri dishes (3 or 5 cm diameter, 10^6 cells per dish). The cells were incubated in Ham's F10 supplemented with 0.2 % FCS, with [^{14}C]-leucine 0.5 µCi, 0.2 mM.

At the end of the incubation, the medium was removed and the cells immediately dissolved in 1 ml of 0.4 % sodium deoxycholate, 0.1 M NaOH. They were then sonicated 5 seconds at 4°C ; an aliquot was precipitated by the same volume of 20 % trichloracetic acid (TCA), washed twice with 1 ml of 10 % TCA, 1 ml ethanol in order to remove lipids. The pellet was then dissolved in 1 ml of 0.4 % sodium deoxycholate, 0.1 M NaOH for 1 hour at 37°C in order to hydrolyse the tRNAs, reprecipitated by an equal volume of 20 % TCA, then dissolved in sodium deoxycholate. An aliquot was used to count the radioactivity and proteins were measured on another aliquot according to Lowry (11). Each experimental point was done in triplicate.

To study the electrophoretic protein profile of the cells, 5×10^6 cells were seeded in 25 cm^2 Falcon flasks, and after 48 hours they were incubated with 2 µCi/ml [^{3}H]-leucine, 0.1 mM, in HAM's F10 supplemented with 0.2 % FCS. After various incubation times, the cells were rinsed twice with Ham F10 medium and solubilized in a 50 mM Tris-HCl pH 6.8 buffer containing 1 % sodium dodecyl sulfate (SDS), 1 mM ethylene dinitrilo tetraacetic acid (EDTA). One ml of the cell suspension was then dialyzed twice for 2 hours against one liter of the same buffer containing 0.1 % SDS. 20 % acrylamide gels were prepared according to Neville et al (12). We used the J3561 gel system which runs and stacks at pH 7.22. The upper reservoir contained 0.1 % SDS. For each series of experiments the amount of protein subjected to electrophoresis was the same (between 50 to 100 µg of solubilized proteins).

After electrophoresis, the gels were cut in slices of 2 mm with a Gilson aliquogel fractionator. Each slice was dissolved in 450 µl of 0.4 % sodium deoxycholate and counted in 5 ml of scintillation fluid (8.25 g of Scintimix, 500 ml of triton X 100, in 1 l of Toluene).

20αOH progesterone was measured in triplicate by a radioimmunoassay (antibodies of E.G. Abraham : S 1556 # 5) following a previously described method (13) except that the steroid was measured directly in the medium, without prior fractionation on a celite column.

TABLE I

Incorporation of ^{14}C -leucine into TCA insoluble proteins

	[^{14}C]-leucine incorporated into TCA insoluble proteins : cpm/µg proteins	20αOH progesterone ng/ml of medium
Controls	1739 ± 62	12 ± 1
DbcAMP 1 mM	1827 ± 63	78 ± 5 *
cAMP 1 mM	1646 ± 36	69 ± 4 *
$ACTH_{1-24}$ 10^{-6} M	2251 ± 60 *	80 ± 6 *
Cholera toxin 1 µg/ml	1638 ± 40	85 ± 5 *
$NPS\text{-}ACTH_{1-24}$ $5 . 10^{-6}$ M	1720 ± 50	46 ± 3 *
$ACTH_{11-24}$ $2 . 10^{-5}$ M	1670 ± 42	12 ± 1

Cells were incubated for 7 hours as described under Methods. The data represent the mean values ± SD of 3 different experiments (in each experiment, each point was done in triplicate).

* significant as compared to controls $p < 0.001$.

RESULTS

INCORPORATION OF ^{14}C -LEUCINE INTO TCA-INSOLUBLE PROTEINS AFTER ACTH STIMULATION

As shown in Table I, ACTH at 10^{-6} M induces a significant increase of [^{14}C]-leucine incorporation into TCA-insoluble proteins. This increase varied from 18 to 30 % in different experiments, and was maximal after seven hours of incubation. Similar stimulation was also found with 10^{-8} M ACTH, but because of the small amplitude of this stimulation, it was difficult to establish a dose-response curve. Neither cholera toxin, nor cyclic AMP in the presence of 3 isobutyl 1 methylxanthin (MIX) (data not shown) nor $NPS\text{-}ACTH_{1-24}$ were able to reproduce the effect of ACTH.

PROTEIN PROFILE OF SOLUBILIZED ADRENAL CELLS AFTER ACTH STIMULATION IN 20 % SDS ACRYLAMIDE GELS

The effects of $ACTH_{1-24}$ on the incorporation of [^{3}H]-leucine into specific proteins are shown in Figure 1. After seven hours of incubation, ACTH 10^{-8} M to 10^{-6} M causes a dramatic and constant increase (50 to 100 %) in the incorporation of a small protein (peak X) of which the rf is 0.80. The migration of 3 different labelled proteins and [^{3}H]-leucine, which were subjected to SDS acrylamide electrophoresis without prior solubilization in 1 % SDS is also indicated. The MW of the protein of peak X is thus about 3500.

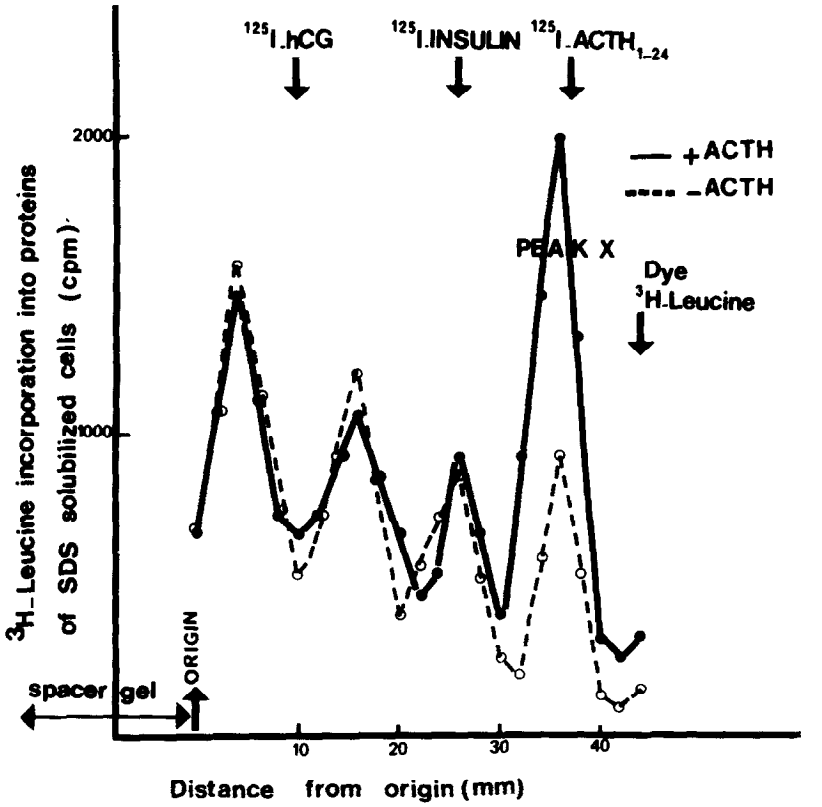

Figure 1 : Effect of ACTH on the incorporation of [^{3}H]-leucine in proteins fractions of adrenal cells.

Cells were incubated for 7 hours in the presence or absence of 10^{-8} M ACTH. They were then solubilized and dialyzed as described under Methods. They were then subjected to 20 % SDS acrylamide gel, protein staining and quantitation. The radioactivity in the gel in the presence or absence of ACTH is plotted as a function of distance from the origin. The results of the experiment shown are representative of 10 separate experiments.

$ACTH_{1-24}$ and hCG were labelled as previously described (14, 15), ^{125}I-insulin was purchased from the Radiochemical Centre, Amersham, England.

Since adrenodoxin was recently reported to be increased in these cell lines after ACTH stimulation (9) we solubilized pure bovine adrenodoxin (a gift from Dr. Kimura) with 1 % SDS and then subjected it to 20 % SDS acryla-

TABLE II

Lack of effect of ACTH in the presence of unlabelled leucine

Preincubation (hrs) with $[^3H]$-leucine	Incubation with unlabelled leucine 5 mM	cpm incorporated in peak X
2 hours	Controls 7 hours	1936 ± 40
	ACTH 10^{-8} M 7 hours	1850 ± 50
15 hours	Controls 7 hours	5927 ± 300
	ACTH 10^{-8} M 7 hours	5545 ± 200

The cells were incubated either two or 15 hours in the presence of 1 µCi/ml of 0.2 mM $[^3H]$-leucine. They were then rinsed twice with HAM F10 medium containing 5 mM leucine and incubated in this medium for 7 hours in the presence or absence of ACTH. The cells were then treated as described under Methods and subjected to 20 % SDS acrylamide gels.

The data represent the mean values ± SD of two different experiments.

mide gel : two main bands were seen whose rfs were 0.50 and 0.60.

We also measured the effect of alkaline hydrolysis on the amount of radioactivity incorporated in the peak. After a stimulation of 7 hours by 10^{-8} M ACTH in the presence of 2 µCi/ml of $[^3H]$-leucine, the cells were solubilized and dialyzed as described under methods, then incubated for 90 min. in the presence of 0.3 M NaOH. A second dialysis was performed in the same conditions as in the first one, and the cells were subjected to electrophoresis in 20 % SDS polyacrylamide gels. Control samples without alkaline hydrolysis were run simultaneously to the ones that had been submitted to alkaline pH. The radioactivity incorporated in peak X of the controls was 2020 ± 100 cpm, that incorporated in samples subjected to alkaline hydrolysis was 2400 ± 150 cpm.

This makes unlikely the possibility that this peak might be a t-RNA. Alternatively this peptide of small molecular weight could also represent a product of ACTH stimulated degradation of higher MW proteins. In order to rule out this hypothesis cells were first incubated for 2 or 15 hours in the presence of 1 µCi/ml of 0.2 mM $[^3H]$-leucine (Table II). They were then rinsed twice with HAM F10 medium, 0.2 % FCS, 5 mM leucine, and further incubated in the same medium in the presence or absence of ACTH. The lack of increase of the peak in these conditions show that ACTH's increase of peak X is not due to the stimulation of the degradation rate of high MW proteins.

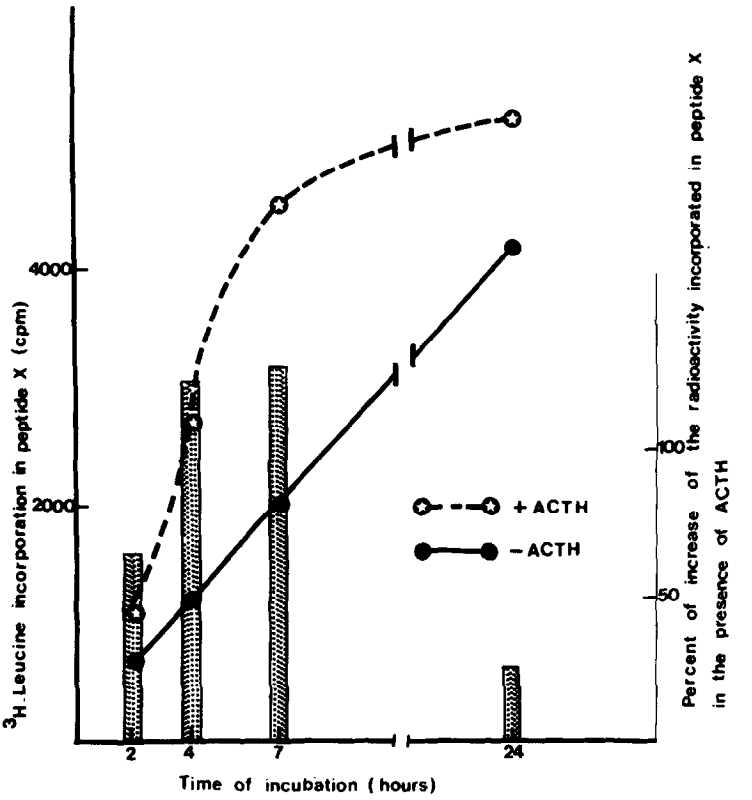

Figure 2 : Time-course of stimulation of the synthesis of peptide X by ACTH.
Cells were incubated for various periods of time with [^{3}H]-leucine 1 µCi/ml in the presence or absence of ACTH. At the end of the incubation, they were treated as described under Methods and subjected to 20 % SDS-acrylamide gels.
The radioactivity incorporated in peak X is plotted as a function of the incubation time. Bars represent the percent of increase of the incorporation of [^{3}H]-leucine in peptide X in the presence of ACTH.
The results are representative of two experiments.

TIME COURSE OF STIMULATION OF THE SYNTHESIS OF PEPTIDE X BY ACTH

Figure 2 shows that ACTH stimulation (64 %) is already significant after 2 hours, then reaches a plateau between 4 and 7 hours. At 24 hours, the stimulation is lower than that found at 2 hours.

EFFECTS OF VARYING THE CONCENTRATION OF ACTH

Table III shows that a small but significant increase of incorporation of [^{3}H]-leucine is already found with 10^{-9} M ACTH. Maximum stimulation is reached with 10^{-8} M ACTH. Roughly, the kinetic of the stimulation seems to follow that of steroidogenesis.

TABLE III

Effect of varying concentrations of ACTH on incorporation of radioactivity into peptide X

Treatment	Radioactivity incorporated in peptide X cpm	20αOH progesterone ng/ml
Controls	1700 ± 50	14 ± 2
ACTH 10^{-9} M	2200 ± 60 *	64 ± 4 *
ACTH 10^{-8} M	3147 ± 100 *	88 ± 5 *
ACTH 10^{-7} M	2600 ± 80 *	95 ± 6 *
ACTH 10^{-6} M	2700 ± 90 *	95 ± 6 *

The cells were incubated for 7 hrs with increasing concentrations of ACTH. At the end of the incubation the medium was removed and assayed for 20αOH progesterone, as described under Methods. The cells were treated as described above and subjected to 20 % SDS acrylamide gels. The data represent the mean values ± SD of two different experiments.

* significant as compared to controls $p < 0.001$.

EFFECT OF OTHER COMPOUNDS

In order to elucidate the mechanism of action of ACTH in this protein synthesis stimulation, we looked at the effects of other compounds which increase cyclic AMP formation and steroidogenesis. Table IV shows that all the compounds which increase steroidogenesis (DbcAMP, cholera toxin, NPS-$ACTH_{1-24}$) also increase the synthesis of peptide X. On the other hand $ACTH_{11-24}$ which binds to adrenal cell membranes (14) but which does not stimulate steroidogenesis does not affect this peptide. Nevertheless, this effect of ACTH is not the result of an increased steroid production since 20αOH progesterone which is the main steroid produced by this cell line is unable to increase the incorporation of [^{3}H]-leucine into the peptide X.

DISCUSSION

In the mouse adrenal cell line Y_1, it has been reported that ACTH has many effects including stimulation of steroidogenesis, inhibition of DNA synthesis (16), and increase of the level of cytochrome P 450 and of adrenodoxin (8, 9). Present data show that ACTH also stimulates total protein synthesis in these adrenal cells, but this effect is not mimicked by other steroidogenic compounds. One could ask whether or not this effect was due to the increase of a specific protein. Our results show for the first time that ACTH is able to

TABLE IV

Effects of steroidogenic compounds on the incorporation of [^{3}H]-leucine into peptide X

Treatment	Radioactivity incorporated in peptide X : cpm	20αOH progesterone ng/ml
Controls	1750 ± 60	22 ± 2
DbcAMP	3250 ± 100	175 ± 10
$ACTH_{1-24}$ 10^{-6} M	3100 ± 98	180 ± 9
Cholera toxin 1 µg/ml	3600 ± 110	192 ± 11
Controls	1564 ± 55	6 ± 0.5
$ACTH_{1-24}$ 10^{-6} M	2528 ± 70	103 ± 9
NPS-$ACTH_{1-24}$ $5 . 10^{-6}$ M	2520 ± 72	30 ± 3
$ACTH_{11-24}$ $2 . 10^{-5}$ M	1600 ± 50	6.5 ± 0.5
20αOH-progesterone 100 ng/ml	1592 ± 53	-

The cells were incubated in the presence or absence of different compounds affecting steroidogenesis.
At the end of the incubation the medium was removed and assayed for 20αOH-progesterone, and the cells treated as described under Methods.
They were then subjected to 20 % SDS acrylamide gels.

induce in the whole cell an increase of the synthesis of a specific peptide (MW 3500). Other reports have dealt with specific proteins found specially in mitochondria (8, 9) but adrenodoxin which is also increased by ACTH is different from peptide X. These reports do not show any correlation between the increase in mitochondrial protein synthesis and total protein synthesis.

Our data show first that peptide X represents roughly 10 % of total protein synthesis. Second, it seems to be dependent upon cyclic AMP while total protein synthesis is increased only by $ACTH_{1-24}$. Therefore the increase of peptide X after ACTH treatment probably cannot account for the stimulation of total protein synthesis observed with the hormone.

The role of this peptide is unknown. Studies are in progress in order to localize the peptide in the cell and to precise its characteristics and function.

ACKNOWLEDGEMENTS

We thank Joëlle Bois for secretarial help. This work was supported by grants from Délégation Générale à la Recherche Scientifique et Technique and Institut National de la Santé et de la Recherche Médicale (ATP n° 40-76-72).

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