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ACTH IN VIVO STIMULATION OF THE SYNTHESIS OF A SPECIFIC MITOCHONDRIAL PROTEIN IN THE RAT

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SUMMARY

Protein markers induced by hormones are the necessary probes to study hormone regulation of gene expression. We recently showed that ACTH was able to induce one of these markers in the cytosol of the rat adrenal (Dazord et al, Biochem. J. 176, 233-239, 1978). In this paper we described another protein marker whose localization is mitochondrial and whose MW is 134 K. Maximal stimulation is seen 2 hours after ACTH injection. Actinomycin D injected 30 min before or 1 hour after the hormone blocks the stimulation. In hypophysectomized rats both ACTH and cyclic AMP are able to stimulate the synthesis of this protein.

ACTH does not only stimulate adrenal steroidogenesis, but also increases in vivo adrenal protein synthesis (1).

Further progress in understanding the mechanisms by which the hormone exerts its tropic effect has been hindered until the recent years, by the failure to detect suitable protein markers induced by the hormone. We have already shown that ACTH administration stimulates the synthesis of a specific adrenal cytosolic protein (2). The effect of the hormone, which is mediated by cyclic AMP seems to take place at the transcriptionnal level (3). In the present paper we describe another protein, whose localisation is mitochondrial, and whose synthesis is specifically stimulated by ACTH.

MATERIALS AND METHODS

Chemicals. The corticotropin used in these studies was Synacthen corticotropin (1-24)-tetracosapeptide -Synacthene Immediat and Retard were purchased from CIBA. [^{3}H]-leucine (specific activity 25 to 35 Ci/mmole) and [^{14}C]-leucine (specific activity 40 to 55 mCi/mole) were obtained from Saclay, France. Acrylamide and N,N'-methylene bisacrylamide of electrophoresis purity were from Polysciences.

Animals. Male Sprague-Dawley rats aged 2-3 months, were used in all experiments (0.2-0.3 kg body wt). They were injected intramuscularly simultaneously with 20 µg of Synacthen Retard and 25 µg of synacthene Immediat, or only with 50 µg Synacthene Immediat and killed by cervical dislocation at various times after the

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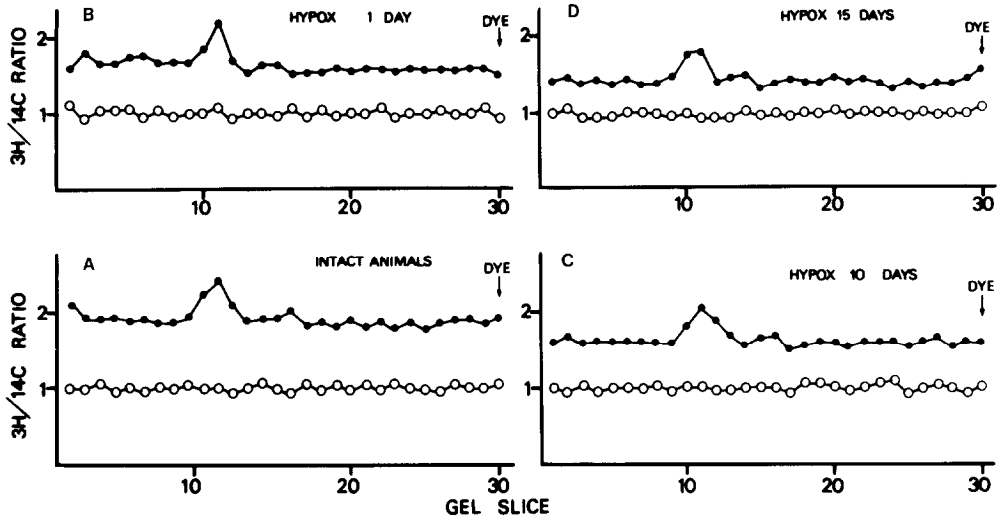

Figure 1. $^{3}H/^{14}C$ ratios of the labelled proteins from control (O) and ACTH-treated animals (●).

ACTH-treated animals received 25 µg Synacthen Immediat and 25 µg Synacthen Retard and were killed 5 hours later. Adrenal slices from controls were incubated separately with either [^{3}H] or [^{14}C] leucine. The same amount of proteins (100 µg) labelled with [^{3}H] or [^{14}C] leucine were coelectrophoresed in 11 % (w/v) polyacrylamide gels in the presence of 0.1 % SDS (O). Adrenal slices from ACTH-treated animals were incubated with [^{3}H] leucine and then mitochondrial proteins were coelectrophoresed with those of controls whose incubations had been carried out with [^{14}C] leucine (●). The gels were cut into 1 mm slices and the $^{3}H+^{14}C$ ratio was determined in each slice.

injection. Control animals received saline. Hypophysectomized rats were purchased from IFFA-CREDO, France. Hypophysectomy was performed by transaural approach and its adequacy was confirmed by examination of the sella turcica and measurement of plasma corticosterone and prolactin as previously described (3).

Radioactive leucine incorporation into adrenal slices was performed as previously described (2).

Isolation of mitochondria. At the end of the incubation period the adrenal slices were homogenized with a polytron PT_{10} in 25 mM Tris/HCl, 2 mM $MgCl_2$, 250 mM sucrose and centrifuged twice at 800 g, 10 min, at 4°C. The resultant supernatant was centrifuged for 10 min at 10,000 g in a refrigerated RC_3 Sorvall centrifuge. The pellet was resuspended in the same conditions. The supernatant was discarded and the pellet solubilized in 25 mM tris-HCl pH 7.4 containing 1 % sodium dodecyl sulfate (SDS). Before electrophoresis dithiothreitol (DTT) was added to the sample to a final concentration of 100 mM, the samples were boiled for 5 minutes then heated at 60°C for 1 hour. Further washes of the pellet do not modify their protein composition. Therefore it was assumed that the proteins analysed in these subcellular fractions were mitochondrial.

Electrophoresis. Electrophoresis was carried out using two systems : the previously described (2) dual labelling technique in gel tubes or alternatively slab gels with the same gel system (4) but with the apparatus designed by Madjar et al (5). The gels were stained for proteins, then processed for fluorography according to Laskey et al

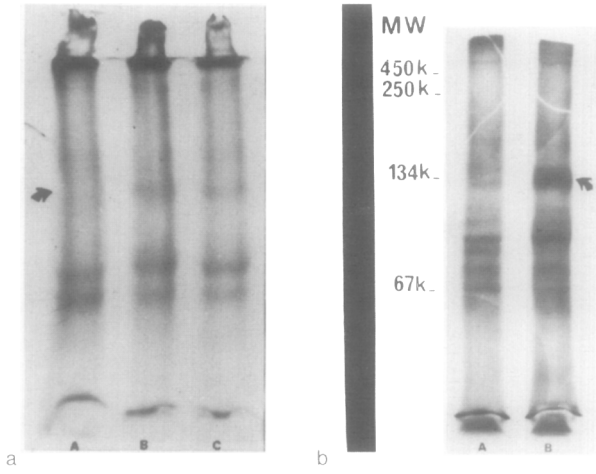

Figure 2.a. Autoradiograms of adrenal mitochondrial proteins of 1 day hypophysectomized rats.
Channel A : controls.
B : ACTH-treated animals (see legend of figure 1).
C : dibutyryl cyclic AMP-treated animals (the animals received eight intramuscular injections of 10 mg at 30 min intervals).

b. Autoradiogram of mitochondrial proteins of intact animals.
Channel A : control animals.
B : ACTH-treated animals (see legend of figure 1).
The standards are Ferritin 450K, Catalase 250K, bovine serum albumin dimer 134K, bovine serum albumin 67K.

(6), or impregnated with sodium salicylate according to Chamberlain (7). The gels were then exposed to Kodak X-Omat XR-1 films at - 70°C for 2 days.

RESULTS AND DISCUSSION

Radiolabelled mitochondrial proteins were analyzed by SDS gel electrophoresis using 2 different techniques. In the first set of experiments, adrenal slices from ACTH-treated rats were labelled with $[^3H]$ leucine while proteins from controls were labelled with $[^{14}C]$ leucine. When the samples were ready for electrophoresis, the mitochondrial proteins labelled with $[^3H]$ leucine were mixed with the same amount of proteins labelled with $[^{14}C]$ leucine (Fig. 1, upper curves). In control experiments, adrenal slices from non-treated animals were labelled separately with either $[^3H]$ or $[^{14}C]$ leucine. The mitochondrial proteins labelled with the 2 different isotopes were coelectrophoresed (Fig. 1, lower curves). As expected ACTH produces an overall increase of the $^3H/^{14}C$ ratio in each slice as shown by the comparison of the upper and lower curves of figure 1A. However if one considers the upper curve, a distinct increase in the $^3H/^{14}C$ ratio over baseline was detected at Rf 0.33 (Protein M). These data are confirmed by autoradiograms fig. 2B. The MW of the 0.33 Rf protein estimated by comparison with the mobilities of known molecular

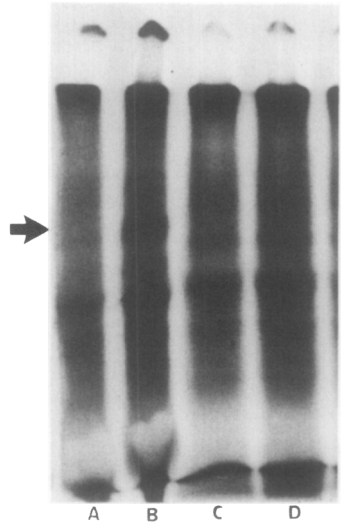

Figure 3. Autoradiograms of ^{3}H-labelled adrenal and kidney mitochondrial proteins.
Channel A : Adrenal ; control animals
B : Adrenal, ACTH-treated animals
C : Kidney, control animals
D : Kidney, ACTH-treated animals.
The arrow indicates the place of the 134 K standard.

weight standards was of 134 000 daltons (This band was cut out of the gel, shown to have the highest $^{3}H/^{14}C$ ratio, and to represent roughly 1 % of the labelled mitochondrial proteins).

The stimulatory effect of ACTH on the synthesis of protein M was also observed in rats which were hypophysectomized 1 to 15 days before the experiment (fig. 1B, 1C, 1D). The effect seems to be mediated by cyclic AMP, since after the administration of dibutyryl cyclic AMP to hypophysectomized rats (fig. 2A), a significative stimulation was observed. On the other hand this effect is not observed in non target tissues of ACTH, like the kidney (fig. 3) or the heart (data not shown).

Steroids are not responsible for the action of ACTH. Aminoglutethimide at doses that completely block ACTH stimulated steroidogenesis (24 mg intramuscularly 3 h before corticotropin treatment and 16 mg intramuscularly at the time of corticotropin injection) does not suppress the effects of ACTH on protein M synthesis (results not shown). The fact that stimulation of protein M is not mediated by steroids is further confirmed by the fact that dexamethasone at a concentration sufficient to suppress endogenous ACTH secretion (as shown by the decrease in plasma corticosterone concentration) (40 μg intramuscularly 30 min. before corticotropin and 40 μg at the time of ACTH injection) has no effects by itself on protein M

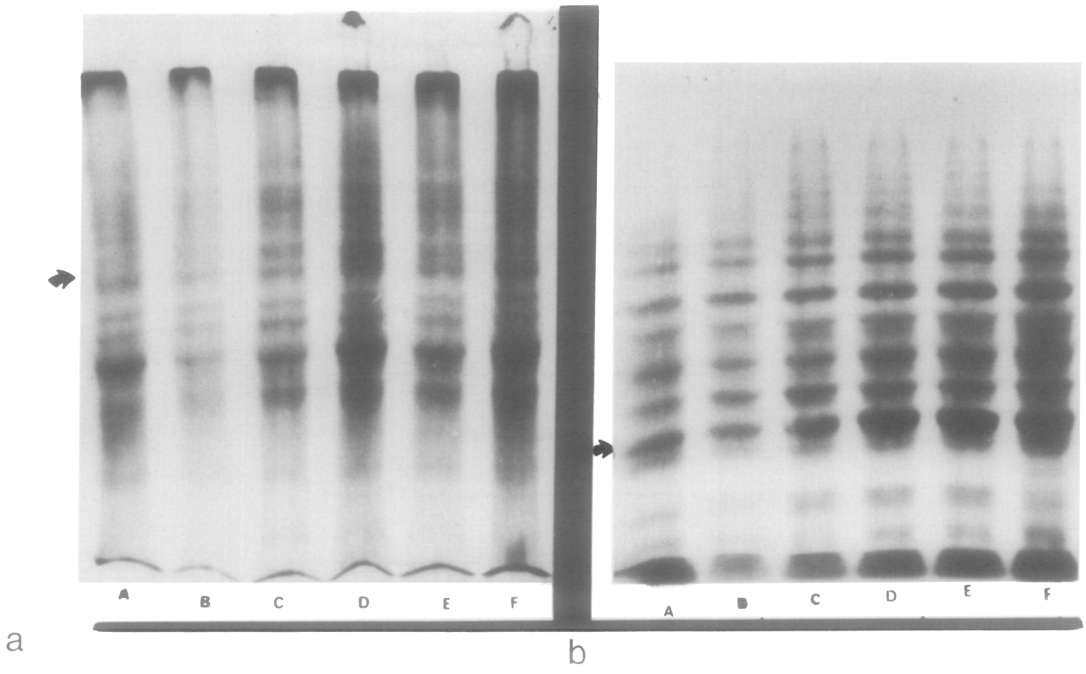

Figure 4. a. Autoradiograms of adrenal ^{3}H-labelled mitochondrial proteins.
A : controls
B : 30 minutes after a single injection of 50 µg of Synacthen Immediat
C : 1 hour after
D : 2 hours after
E : 4 hours after
F : 7 hours after
In all channels 80 µg of proteins were submitted to electrophoresis except in channel A and F where 120 µg were electrophoresed.
The arrow indicates the place of the 134K standard.

b. Autoradiograms of adrenal [^{3}H]-labelled cytosolic proteins.
A : controls
B : 30 minutes after a single injection of 50 µg of Synacthen Immediat
C : 1 hour after
D : 2 hours after
E : 4 hours after
F : 7 hours after.
In all channels 80 µg of proteins were submitted to electrophoresis.
The arrow indicates protein E (see Dazord et al, (2)).

synthesis and does not modify ACTH stimulation of protein M synthesis (results not shown).

In order to estimate the time course of the stimulation of protein M, the animals were killed at different times after a single injection of 50 µg of Synacthen Immediat. As it is shown in figure 4a, it is obvious that 2 proteins migrate in the 134K area. ACTH stimulates only the upper band, which is barely visible in control conditions or 7 hours after ACTH injection. Furthermore in both cases, more

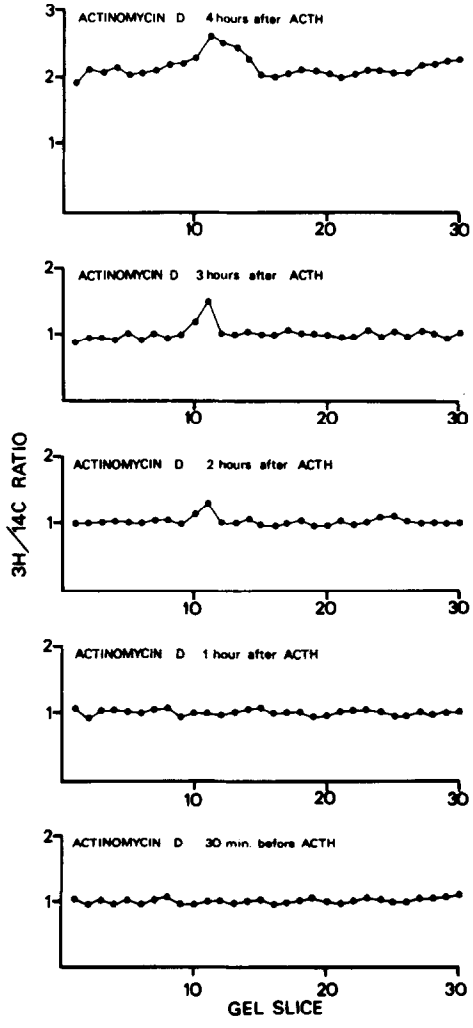

Figure 5. Effects of actinomycin D on adrenal protein M synthesis, when injected at different times before or after ACTH.
Actinomycin D (500 µg) dissolved in methanol was injected at different times before or after ACTH treatment (25 µg Synacthen Immediat and 20 µg Synacthen Retard) and the rats killed 5 hours after ACTH injection.
Control animals (^{14}C-labelled) received neither ACTH nor Actinomycin D.

proteins were submitted to electrophoresis. A slight stimulation is already observed 1 hour after ACTH administration, and reaches a maximum at 2 hours. Thereafter a progressive decline is observed. This time course is quite different from that observed for the cytosolic protein E, which reaches a maximum at 4 hours, and remains elevated 7 hours after ACTH administration (figure 4b).

To obtain more information about the mechanisms involved in the control of protein M synthesis the effects of Actinomycin D were studied. As shown in figure 5, stimulation of protein M synthesis is completely prevented when Actino-

mycin D is injected 30 min before or 1 hour after ACTH. The protein starts to escape to Actinomycin D inhibition when the antibiotic is injected 2 hours after ACTH, and regains its normal value when it is injected 3 or 4 hours after the hormone. It is interesting to note that when actinomycin D is injected 2 or 3 hours after ACTH, it blocks the bulk stimulation of protein synthesis induced by ACTH, as attested by the baseline of the $^{3}H/^{14}C$ ratio, while the effect of the hormone on protein M synthesis is observed. Similar discrepancy between the regulation of protein E and total cytosolic protein synthesis has also been observed (3).

Like for the recently described protein E we do not know the identity or nature of protein M. Likewise the role of both these proteins is still unknown. However considering the time course of the ACTH stimulation, it is unlikely that they are implicated in the stimulation of steroidogenesis by ACTH or rather, they could be involved in the tropic action of the hormone. If they are present in the abundance indicated by our preliminary findings, 1 % of the mitochondrial proteins for protein M, and 2 % of the cytosolic proteins for protein E they may be specific probes needed to permit isolation of hormone regulated message and to further study ACTH regulation of gene expression in the adrenal.

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