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THE NEUROTROPIC ACTION OF ACTH: EFFECTS OF ACTH-LIKE PEPTIDES ON THE INCORPORATION OF LEUCINE INTO PROTEIN OF BRAIN STEM SLICES FROM HYPOPHYSECTOMIZED RATS

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SUMMARY

The present study reports on the effects of behaviorally active, N-terminal fragments of ACTH on the incorporation rate of leucine into proteins in slices taken from the brain stem of hypophysectomized rats. In parallel with *in vivo* studies, the presence of $ACTH_{1-24}$ or $ACTH_{1-10}$-7-*l*-Phe in the incubation medium stimulates the incorporation of [^{14}C]leucine into protein, whereas $ACTH_{11-24}$ is ineffective. No changes are found with $ACTH_{1-10}$-7-*d*-Phe, the analogue which exerted opposite effects both in behavioral situations and on leucine incorporation *in vivo*. These data suggest a direct action *in situ* of ACTH on central nervous structures. The sequence 1—10, which lacks appreciable corticotropic activity, appears to be sufficient to exert this neurotropic action of ACTH.

To study the effects of ACTH on rat brain structures, hypophysectomized rats were used. Three weeks after the surgery, these animals provided us with a model in which the experimentals are depleted in corticosteroids and ACTH. There is a lower level of RNA and protein metabolism both in peripheral organs of these animals [7], and in structures of the brain stem as measured *in vivo* [18] and *in vitro* [12]. Hypophysectomized rats are inferior to normals in acquiring a conditioned avoidance response in the shuttlebox [4,5]. Treatment with ACTH or N-terminal fragments of ACTH (*e.g.* $ACTH_{1-10}$) which lack adrenocorticotropic activity, restores the performance in the shuttlebox [5] and increases the incorporation of [^{3}H]leucine into brain stem proteins measured 5 min after injection of the percursor into the diencephalon [15]. Opposite effects of $ACTH_{1-10}$ have been found by substitution of *d*-phenylalanine in the 7th position, both in the behavioral situation [1,6] and on the leucine incorporation *in vivo* [15], whereas

$ACTH_{11-24}$ has no effects [6]. Implantation and lesion studies suggest an important role of the posterior thalamus (nucleus parafascicularis) in the expression of the behavioral effects of N-terminal ACTH fragments [2,16,17]. In brain slices containing these structures from hypophysectomized rats, it has been found that the incorporation of leucine into protein is enhanced by addition of $ACTH_{1-10}$ in concentrations of 10^{-5}, 5×10^{-7} and 10^{-8} M [12].

The present communication extends our studies on *in vitro* incorporation of leucine and reports on the effects of the ACTH analogues $ACTH_{1-24}$, $ACTH_{11-24}$ and $ACTH_{1-10}$-7-*d*-Phe, as compared to $ACTH_{1-10}$-7-*l*-Phe [12].

Male Wistar rats, weighing approximately 110—120 g, were used. Hypophysectomy was performed via the transauricular route under anesthesia with ether. Loss of body weight, adrenal atrophy and macroscopic inspection of the sella turcica were used as indications that hypophysectomy had been performed successfully. Three weeks after the operation the animals were sacrificed by decapitation. A brain slice of about 1 mm thickness was prepared as described elsewhere [12], containing posterior thalamic tissue including the nucleus parafascicularis. The slice was weighed, preincubated for 30 min at 37°C in 2 ml Krebs phosphate medium [8] (containing, in mM: NaCl, 124: KCl, 6.7; $MgSO_4$, 1.3; $CaCl_2$, 0.75; Na_2HPO_4, 20; glucose, 10; adjusted with 2 M HCl to pH 7.4; equilibrated with pure O_2) prior to addition of about 1 μCi of *l*-[U-^{14}C]leucine (311 or 348 mCi/mmole; Radiochemical Centre, Amersham, G.B.) and then incubated for 30 min under the same conditions. At the end of the incubation the slice was blotted with filter paper and homogenized in 1.5 ml of ice-cold medium containing unlabeled leucine (10 mM). In order to measure the radioactivity incorporated into proteins, duplicate samples of the homogenate plus 5 mg albumin carrier protein were treated with an equal volume of 1 M perchloric acid at 0°C and centrifuged. The precipitates containing the labeled proteins [15] were washed 3 times with 0.5 M perchloric acid, transferred to scintillation vials, solubilized using approximately 1 ml of a tissue solubilizer (Soluene-350, Packard Instr. Co.) and [^{14}C] radioactivity was measured in a liquid scintillation counter (Searle, Mark II). As a measure of the intracellular uptake of the [^{14}C] leucine from the medium, the total radioactivity of the homogenate was corrected for that in the inulin space [9]. The inulin space was determined [3] in a separate experiment by incubating slices in a medium containing 0.04% [^{14}C] carboxyinulin (7.7 mCi/mmole; Radiochemical Centre, Amersham, G.B.) instead of leucine, under similar conditions to those described. The correction for extracellular free amino acid was found to be small (about 10% of the total radioactivity in the slices) and appeared not to be affected by the presence of the ACTH peptides.

Slices were taken from the brain stem of hypophysectomized rats and incubated with one of the N-terminal fragments of ACTH as described above. After 30 min preincubation [^{14}C] leucine was added and the incubation was continued for another 30 min (the incorporation period). The results are shown in Table I. Some variations between experiments were noticed, possibly

TABLE I

EFFECT OF ACTH-LIKE PEPTIDES ON THE INCORPORATION AND UPTAKE *IN VITRO* OF [U-^{14}C]LEUCINE IN BRAIN STEM SLICES OF HYPOPHYSECTOMIZED RATS[a]

n = number of rats (incubations). Results are means ± S.E.M.

	n	Radioactivity (dpm/mg tissue)[d]	
		In protein[e]	In slice[f]
Control	8	217 ± 17	3577 ± 122
$ACTH_{1-24}$ 10^{-5} M	7	305 ± 13[b]	3870 ± 199
Control	9	307 ± 22	3600 ± 198
$ACTH_{11-24}$ 10^{-5} M	9	300 ± 20	3792 ± 182
Control	9	271 ± 22	3524 ± 251
$ACTH_{1-10}$-7-*l*-Phe 10^{-5} M	9	384 ± 49[c]	3602 ± 218
Control	9	313 ± 25	3501 ± 119
$ACTH_{1-10}$-7-*d*-Phe 10^{-8} M	7	326 ± 33	3453 ± 132
5×10^{-7} M	8	322 ± 34	3618 ± 342
10^{-5} M	8	296 ± 23	3291 ± 102

[a] Preincubation during 30 min and incorporation during 30 min.
[b] $P < 0.01$ (Student *t*-test).
[c] $P = 0.05$ (Student *t*-test).
[d] Wet weight.
[e] Radioactivity recovered from the acid insoluble precipitate.
[f] Total radioactivity recovered from the slice.

as a result of the use of various batches of [U-^{14}C]leucine with different specific activities. In control animals, the incorporation appeared to be linear up to a 60 min incorporation period, as found previously for similar slices of sham-operated rats [12].

Comparisons of each experimental group with its control show significant increases in the incorporation rate of leucine into protein in the presence of $ACTH_{1-24}$ and $ACTH_{1-10}$-7- *l*-Phe, without changing the uptake into the non-inulin (intracellular) space (Table I). No effects were observed with $ACTH_{11-24}$. The results so far are consistent with the *in vivo* findings by Schotman *et al.* [15] (see above), and with the reported stimulation of protein synthesis in mouse brain by melanotropic peptides sharing the sequence $ACTH_{4-10}$ [13]. The observed increase in incorporation *in vivo* is not likely to be due to changes in size or metabolism of the precursor pool [10,11,13—15]. Furthermore, under the *in vitro* conditions used in this paper, eventual systemic effects of ACTH can be excluded. Moreover, under these *in vitro* conditions, no changes were observed in the uptake of leucine into the extracellular (inulin) space (this paper) and in the uptake of leucine or its metabolically inert analogues

α-aminoisobutyric acid and cycloleucine (2-aminocyclopentane) into the cells (Schotman, unpublished). Thus, the present paper further substantiates the hypothesis that brain protein synthesis is indeed affected by ACTH-like peptides, although an effect on pool compartmentation cannot be excluded. The lack of effect *in vitro* of $ACTH_{1-10}$-7-*d*-Phe, which *in vivo* showed an effect on leucine incorporation opposite to that of $ACTH_{1-10}$-7-*l*-Phe, suggests a difference in the mode of action between both peptides. At present we cannot account for this discrepancy; further work is in progress to elucidate the mechanism of action of this peptide.

The combined data so far on the *in vivo* and *in vitro* effects of $ACTH_{1-10}$-7-*l*-Phe on leucine incorporation into protein and the lack of effect on RNA metabolism [15] support the notion that the site of action of this peptide is at the translational level [7].

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