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THE ACTIVE SEQUENCE IN THE ACTH MOLECULE RESPONSIBLE FOR INHIBITION OF THE EXTINCTION OF CONDITIONED AVOIDANCE BEHAVIOUR IN RATS

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The effect of structural analogues of the N-terminal decapeptide of ACTH on inhibition of extinction of a conditioned avoidance response in rats has been studied. Studies involving the relation between chain length and behavioural activity revealed that the sequence 4-10 is the shortest peptide which affects the avoidance response as potently as does the decapeptide. Although the presence of the sequence 1-3 is not essential for the behavioural effect, slight structural modifications within these 3 amino acids are accompanied by loss of activity.

ACTH-like peptides Structure-activity studies Conditioned avoidance behaviour

1. INTRODUCTION

In a previous study (De Wied, 1966) ACTH and related peptides were shown to inhibit extinction of a shuttle-box and a pole jumping avoidance response in rats. When further fragments of ACTH became available, a follow-up study could be undertaken to determine the part of the ACTH molecule responsible for this behavioural effect.

2. METHODS

Male white rats were conditioned to jump onto a pole (De Wied, 1966). The conditioned stimulus (CS) was a light emitted by a 60 Watt bulb presented for 5 sec. A rat which did not jump onto the pole but remained on the grid floor of the cage received an electric shock (25V; 5mA) which served as an unconditioned stimulus (US). Ten conditioning trials were given each day for 3 days with an inter-trial interval averaging 60 sec. Rats showing positive responses in 10 or more of these 30 conditioning trials were used for extinction trials. Extinction was studied over the next 3 consecutive days using the same procedure as that employed for learning, except that the US was not presented.

The total number of positive conditioned avoidance responses (CAR's) scored by each rat during either learning or extinction served as an index of avoidance behaviour. The significance of differences between groups was determined with the aid of Wilcoxon's two sample test (Wilcoxon, 1945). ACTH peptides were administered in the form of long-acting zinc phosphate preparations (De Wied, 1966) to various groups of rats conditioned in this manner. Treatment was started on the third day of the learning period immediately following training. A single subcutaneous injection of 10 μg of each peptide was administered. ACTH 1-10 peptide was used as a reference preparation. This peptide exhibits a behavioural effect similar to that of synthetic ACTH 1-24 (De Wied, 1966).

3. RESULTS

Table 1 summarizes the results. It is clear that ACTH 4-10 is the shortest amino acid sequence possessing an inhibitory effect on extinction of the CAR identical to that exerted by ACTH 1-10. This heptapeptide is probably the active part of the ACTH molecule responsible for the inhibitory effect on extinction of avoidance behaviour. The sequence ACTH 5-10 also showed a significant inhibition of extinction although the effect was not as strong as that of ACTH 4-10. It should however be borne in mind that, as shown

Table 1
Effect of fragments of ACTH on the rate of extinction of a pole jumping avoidance response in rats.

	Amino acid sequence										Number of conditioned avoidance response		
	1	2	3	4	5	6	7	8	9	10	Learning	Extinction	
ACTH 1-10	H-	Ser-	Tyr-	Ser-	Met-	Glu-	His-	Phe-	Arg-	Trp-Gly-OH	14 ± 0.7 *	20 ± 1.0 ‡	(8)
1-β-Ala-ACTH 1-10	H-	β-Ala-	Tyr-	Ser-	Met-	Glu-	His-	Phe-	Arg-	Trp-Gly-OH	14 ± 0.6	14 ± 1.0	(10)
1-Thr-ACTH 1-10	H-	Thr-	Tyr-	Ser-	Met-	Glu-	His-	Phe-	Arg-	Trp-Gly-OH	15 ± 0.6	11 ± 1.5	(10)
2-D-Tyr-ACTH 1-10	H-	Ser-	D-Tyr-	Ser-	Met-	Glu-	His-	Phe-	Arg-	Trp-Gly-OH	14 ± 0.8	14 ± 0.9	(9)
8-Lys-ACTH 1-10	H-	Ser-	Tyr-	Ser-	Met-	Glu-	His-	Phe-	Lys-	Trp-Gly-OH	14 ± 0.7	21 ± 0.7 ‡	(9)
ACTH 2-10		H-	Tyr-	Ser-	Met-	Glu-	His-	Phe-	Arg-	Trp-Gly-OH	15 ± 0.4	18 ± 1.2 ‡	(9)
ACTH 3-10			H-	Ser-	Met-	Glu-	His-	Phe-	Arg-	Trp-Gly-OH	15 ± 0.4	21 ± 0.8 ‡	(8)
ACTH 4-10				H-	Met-	Glu-	His-	Phe-	Arg-	Trp-Gly-OH	15 ± 0.4	23.5 ± 0.4 ‡	(8)
ACTH 5-10					H-	Glu-	His-	Phe-	Arg-	Trp-Gly-OH	15 ± 0.6	17 ± 1.0 ‡	(11)
ACTH 6-10						H-	His-	Phe-	Arg-	Trp-Gly-OH	15 ± 0.5	13 ± 0.9	(12)
ACTH 7-10							H-	Phe-	Arg-	Trp-Gly-OH	16 ± 0.5	14 ± 1.1	(10)
Placebo											15 ± 0.2	12 ± 0.5	(30)

* Mean ± standard error of the mean.
( ) Number of animals.
‡ Significant difference from the controls ($p < 0.05$).

in previous experiments, the behavioural effect of ACTH and related peptides under these experimental conditions could be elicited only if long acting preparations were used. Since complex formation with zinc phosphate may depend on the length of the peptide chain, it is possible that small peptides failed to form efficient complexes and were therefore rapidly absorbed from the subcutaneous tissue. However, in previous experiments (De Wied, 1966) ACTH 5-10 was also less active than the decapeptide ACTH 1-10 even if the treatment was repeated every day during extinction. Accordingly, the lesser activity of the sequence 5-10 is probably not due to inefficient complex formation.

It was further found that the replacement of arginine by lysine in the ACTH 1-10 peptide did not affect its behavioural activity. However, replacement of serine by β-alanine or threonine, or replacement of L-tyrosine by D-tyrosine markedly interfered with potency. Such modifications resulted in an almost complete loss of activity.

For a few of the peptides studied here, the *in vitro* fat mobilizing activity on rabbit adipose tissue was estimated by a modification of the method of Tanaka, Pickering and Li (1962), the non-esterified fatty acids in the medium being determined by the method of Trout, Estes and Friedberg (1960). It was found that, as compared with ACTH 1-10, replacement of L-tyrosine by D-tyrosine did not alter its lipolytic potency, whereas replacement of L-phenylalanine by D-phenylalanine led to a fivefold increase in potency. Potency ratio: ACTH 1-10: 1; 2-D-Tyr-ACTH 1-10: 1.1 (95% fiducial limits: 0.6-2.1); 7-D-Phe-ACTH 1-10: 5.0 (2.6-10.6).

4. DISCUSSION

The effects of two types of analogues of the N-terminal decapeptide of ACTH have been studied on the extinction of conditioned avoidance behaviour in rats. On the one hand the relation between chain length and biological activity was investigated in studies involving the progressive shortening of the peptide chain at the amino end; on the other hand, the effect of slight structural modifications has been examined. Our results compare well with those of Ferrari, Gessa and Vargiu (1963), who reported that the stretching syndrome as induced by intracysternally injected ACTH, α- or β-MSH can still be evoked with the heptapeptide 4-10.

The fact that in the series of peptides with decreasing chain length the sequence 4-10 appears to be the shortest peptide capable of exerting as potent an inhibition of extinction as the decapeptide is reminiscent of similar observations concerning other extra-adrenal activities of these peptides, such as melanocyte-expanding or lipolytic activity. Although there is a fairly wide divergence in the melanocyte stimulating activities reported by different groups of authors (Kastin et al., 1965; Ney et al., 1965; Schröder and Lübke, 1966), the conclusion seems justified that a reduction in the chain length from 4-10 to 6-10 results in a considerable loss of biological activity. The same holds true for the

in vitro lipolytic activity measured in terms of the minimal effective dose when using rabbit adipose tissue. Although the values given in the literature (Tanaka et al., 1961; Raben et al., 1961) show a considerable scatter, they do point to the same conclusion, i.e. that a sharp decrease in activity occurs in the region 4-10 to 6-10.

However, the changes in potency of the three extra-adrenal activities, i.e. the effects on avoidance behaviour, on melanocytes or on lipolysis do not necessarily run parallel for the peptides studied in this investigation. Indeed, the spectrum of biological activities of some structural analogues of the 1-10 decapeptide show that there are divergences in their individual potencies with variations in structure. For example, inversion of configuration of the tyrosine residue in position 2 leads to loss of activity on avoidance behaviour whilst lipolytic activity is retained, whereas replacement of phenylalanine in position 7 by the corresponding D-residue results both in reversal of the action on avoidance behaviour, i.e. facilitation of extinction (Bohus and De Wied, 1966), and in enhancement of lipolytic activity. Dissociation of activity with respect to avoidance behaviour and lipolysis would therefore appear to be feasible. It may be pointed out that the sequence 1-3 can be removed without impairment of the action on avoidance behaviour; it is thus not essential for this type of action. However, slight structural modifications at the N-terminal moiety such as inversion of configuration (from L to D tyrosine), spatial enlargement (from serine to threonine), or alteration in the nature of the terminal amino group (from α to β position) consistently lead to loss of this biological activity. This observation is analogous to certain data obtained for oxytocin. Methylation (Jošt, Rudinger and Šorm, 1963) or acetylation (Boissonnas et al., 1961) of the terminal amino group or inversion of configuration of the cysteine residue in position 1 (Jošt et al., 1963) causes loss of oxytocic activity, whereas absence of the α-amino group results in its potentiation (Hope and Du Vigneaud, 1962).

The results presented here suggest that the identical effects of ACTH, α- and β-MSH on avoidance behaviour may be explained by the fact that these peptides share the sequence 4-10. This may indicate that a relatively small peptide is responsible for the marked behavioural effect of these pituitary hormones.

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