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ISOLATION OF N-ACETYLASPARTIC ACID FROM HYPOTHALAMIC TISSUE AND SIGNIFICANCE OF ITS ACTH-RELEASING ACTIVITY

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SUMMARY

During extensive fractionation of extractives of porcine hypothalami tissue, advanced steps were monitored by RIA for the activity of the corticotropin releasing factor (CRF). Two entities with CRF-activity were observed and identified. They were <GluOH and N-AcAspOH; both released ACTH at μg-levels. AspOH and <GluOH had comparable activity, but AsnOH, GluOH, GlnOH were inactive. N-AcAspOH, and N-AcGluOH, N-AcGlnOH, and <Glu-GluOH showed comparable activity. N-AcAspOH has been identified from brain tissue by many investigators and contemplated as a transmitter. The release of ACTH by N-AcAspOH is a new observation, and allows the concept that an N-AcAsp-peptide might be a hormone for release (or inhibition) of a pituitary hormone including corticotropin.

INTRODUCTION

Since the report in 1957 of a preparation possessing ACTH-releasing activity (corticotropin releasing factor, CRF) (1), many studies have been published concerning this still elusive hormone. Many workers have presumed the peptide nature of CRF (1,2,3,4). Several entities are known to possess CRF activity (2,5), including 5-hydroxytryptamine, vasopressin, pressinoic acid, as well as the cations Cu, Zn, Ni, and ammonia.

We have isolated an amino acid derivative from porcine hypothalamic tissue, and found that it has CRF-activity, in vitro.

Biological Assay Procedure. - The pituitaries for the in vitro assay were removed from 20-day old female rats of the Sprague-Dawley strain. For assay, two pituitaries were incubated at 37°C in 1 ml of lactated Ringer's solution (Travenol Laboratories) in 10 ml-Teflon beakers in a Dubnoff Shaker. After one hour pre-incubation, the medium was discarded and replaced. After the second

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hour of pre-incubation, the medium was removed for assay (P_2) and replaced. The samples for bioassay were added at this time, and incubation was continued for one additional hour, and the medium was removed for assay (I_3). A radioimmunoassay was used for measuring ACTH. The results were calculated by subtraction of the ACTH released at I_3 from the level at P_2. Values are recorded as △pg ACTH/ml medium.

Purification Steps. - About 20 batches of 5,000 lyophilized porcine hypothalamic fragments were homogenized, extracted with 2M acetic acid in methanol, and processed through the following purification steps: gel filtration on BioGel P-2; rechromatography on BioGel P-2; partition chromatography on Sephadex G-25 (11:5:3, 1% acetic acid:n-butanol:pyridine); anion exchange on DEAE-Sephadex; partition rechromatography (11:5:3); chromatography on Sephadex LH-20; partition chromatography on Sephadex G-25 (4:1:5, n-butanol:acetic acid water); HPLC on Poragel-PT; HPLC on Poragel-PS; cation exchange on SP-Sephadex; HPLC on Poragel-PT; gel filtration on Sephadex G-10.

This isolation sequence differed from the one which yielded <GluOH (6) in that there was rechromatography at the BioGel P-2 step, and the step of HPLC with Benzyl-Corasil was omitted.

RESULTS AND DISCUSSION

The fractions for further purification in each next step were selected according to their activities for release or inhibition by several assays including the prolactin and growth hormone assays. The final purification steps were particularly monitored by radioimmunoassay of ACTH release. Two entities with CRF-activity were isolated, No. 1 and No. 2. These two entities were separated by Sephadex G-10 chromatography. No. 1 was strongly retained on G-10 and was essentially pure. No. 2 was less strongly retained than No. 1, and was further purified by adsorption chromatography on silica gel (Baker).

No. 1 was ninhydrin negative indicating no free amino group. Amino acid analysis showed glutamic acid with a small amount of aspartic acid. Field desorption mass spectrometry (FDMS) showed an apparent molecular ion of m/e 130, consistent with the structure of pyroglutamic acid. FDMS of pyroglutamic acid showed an M + 1 at m/e 130. The elution volumes on G-10 for both No. 1 and authentic pyroglutamic acid were identical. The retention times on silica gel tlc in two solvent systems were also identical (11:5:3 upper; R_f No. 1, 0.43; R_F <GluOH 0.43; 4:1:5 upper :5 MeOH; R_f No. 2, 0.38; R_f <GluOH 0.38).

It appeared that all the ACTH-releasing activity in No. 1 was due to <GluOH and not to an active minor constituent.

No. 2, which has a smaller elution volume on Sephadex G-10, was further purified by silica gel chromatography. It was also ninhydrin negative indicating a blocked amino terminus. Amino acid analysis showed aspartic acid with only a trace of glutamic acid. The FDMS showed a molecular weight of m/e 175, which is consistent with that of N-acetylaspartic acid. This assisgnment was confirmed by comparison of No. 2 with N-acetylaspartic acid which showed a molecular ion at m/e 175. Chromatography of No. 2 and N-Ac-AspOH on Sephadex

G-10 resulted in identical elution volumes and identical retention times on silica gel tlc in two different solvent systems.

The in vitro data on <GluOH and N-Ac-AspOH are in Table I. The data on comparison of the sample of No. 2 and synthetic N-acetylaspartic acid indicated, however, that not all of the ACTH-releasing activity was accounted for by N-Ac-AspOH.

This discovery that pyroglutamic acid and N-acetylaspartic acid, which were identified during purification with the guidance of the in vitro assay, release ACTH prompted the investigation of compounds with similar structures. The data are in Table II. <GluOH showed comparable activity to release ACTH. However, asparagine, glutamic acid, and glutamine did not show comparable release of ACTH showing that this effect has some selectivity. After acetylation, GluOH, and AspOH also showed ACTH-releasing activity.

There is an extensive literature, since 1955, on the chemical isolation and biological aspects of N-Ac-AspOH from brain tissue. Tallan et al. (7) in 1956, reported the isolation from whole brain tissue. Other workers confirmed this finding, but a definite physiological function for N-Ac-AspOH remained ambigous. In 1971, Reichelt et al. (8) observed that biogenic amines caused significant decreases in levels of N-Ac-AspOH, in vitro. N-Ac-AspOH appeared to be in neurons, and Reichelt apparently considered possible transmittor roles for acetylated amino acids, since Reichelt and Kvamme (9), in 1967, had found both N-Ac-AspOH and N-Ac-GluOH in mouse and monkey brains.

Our finding that <GluOH and N-Ac-AspOH have activity stimulating CRF may have a direct bearing on the research of other investigators who study the activities of extracts which presumably contain CRF and who work on purification for the ultimate isolation of CRF.

In 1976, Gillies and Lowry (10) investigated CRF using a perfused anterior rat pituitary column. Sayers and Seelig (11) studied the assay and purification of CRF(s) from bovine hypothalami. Jones et al. (12) reported on the purification of CRF from rat hypothalami. Yasuda and Greer (13) studied the intra- and peri-hypothalamic concentration of CRF, and also (14) the relative brain and corporeal concentration of CRF and the correlation with ACTH secretion, and also (15) the distribution of CRF which appeared to have the highest concentration in the hypophyseal stalk of various species.

In 1977, Schally et al. (16) reported on the purification of CRF from porcine hypothalami. Hashimoto and Brodish (17) studied tussue-CRF in plasma by an in vitro bioassay. Maran et al. (18) studied hypothalamic organization for control of ACTH in the cat. Vale, Rivier (19) studied the effects of a putative hypothalamic CRF and known substances on the secretion of radioimmunoassayable ACTH by cultured anterior pituitary cells.

Our finding that N-Ac-AspOH and structurally related compounds release ACTH, although at microgram levels, allows the concept that an N-Ac-Asp-peptide, presently unknown, might be potent for release (or inhibition) of a pituitary hormone including corticotropin.

TABLE I. IN VITRO DOSE RESPONSE EFFECT OF NO. 1 AND NO. 2

	NO.	Dose µg/ml medium I_3	ACTH/RIA Δ pg/ml medium + SEM $I_3-\bar{P}_2$	p value vs A or B
A.	---	---	3 ± 9	---
	1 (< GluOH)	207	93 ± 19	< .01
	1 (< GluOH)	414	142 ± 9	< .001
	1 (< GluOH)	827	242 ± 21	< .001
B.	---	---	-1 ± 4	---
	2 (N-AcAspOH)	370	36 ± 18	< .02
	2 (N-AcAspOH)	666	254 ± 41	< .001

Table II. In Vitro Release of ACTH
By Structurally Related Amino Acids and a Dipeptide

Compound	Dose µg/ml medium	ACTH Δpg/ml ± SEM I_3-P_2	p Value vs 1
-	-	-16 ± 6	-
<Glu	600	234 ± 32	<.001
Asp	600	259 ± 59	<.001
Asn	600	-23 ± 5	ns
Glu	600	-6 ± 3	ns
Glu	1200	11 ± 8	ns
Gln	600	-28 ± 3	ns
N-AcAsp	600	133 ± 16	<.001
N-AcGlu	600	232 ± 32	<.001
N-AcGln	600	102 ± 20	<.001
<Glu-Glu	600	174 ± 20	<.001

Mean (5) I_3-P_2

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