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ISOLATION, STRUCTURAL ELUCIDATION AND SYNTHESIS OF A TETRADECAPEPTIDE WITH IN VITRO ACTH-RELEASING ACTIVITY CORRESPONDING TO RESIDUES 33-46 OF THE α-CHAIN OF PORCINE HEMOGLOBIN

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SUMMARY. A peptide found in acetic acid extracts of porcine hypothalami and capable of stimulating the release of ACTH in vitro was isolated in pure state, structurally identified as Phe-Leu-Gly-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe and synthesized. This tetradecapeptide, which corresponds to amino acid residues no. 33-46 in the sequence of the α-chain of porcine hemoglobin, probably represents an artefact of extraction or isolation procedures. Since this peptide stimulates ACTH release from rat pituitary fragments and from monolayer cultures of pituitary cells, but not in vivo, caution must be exercised in interpreting the results of in vitro assays for corticotropin releasing factor.

Recently we resumed the work on purification of CRF[1] from pig hypothalamic extracts and found several distinct fractions capable of releasing ACTH in vitro (1, 2). One of these materials was isolated in the pure state, identified as Phe-Leu-Gly-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe and synthesized. However, this tetradecapeptide appears to be an artefact of the extraction procedure since it forms part of the α-chain of porcine hemoglobin. This paper reports briefly biochemical and biological characterization of this material.

MATERIAL AND METHODS

The extract from 470,000 pig hypothalami was the same as that used for the isolation of porcine somatostatin (3) and catecholamine (4). All the extraction, purification, composition and structural methods were described in detail previously (3-7). HPLC was carried out using Waters Associates Model 204 apparatus

[1]Abbreviations: CRF, corticotropin releasing factor; HPLC, high pressure liquid chromatography; TLC, thin layer chromatography; MW, molecular weight; CCD, counter current distribution; DANSYL-DNS; K, partition coefficient; CMC, carboxymethyl cellulose; PTH, phenylthiodantoin.

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equipped with a Model UK 6 injector, two Model 6000 A pumps and a Model 660 programmer for gradient elution. The detection of separated components was made with a Schoeffel Model 770 absorbence detector. Chromatography was performed using 4mm x 30cm columns of μ Bonapak C_{18} (10μ). The solvents consisted of mixtures of CH_3CN (Baker glass distilled) in 0.01M NH_4OAc. pH 3.8 buffer. Programmed linear concentration gradients from two reservoirs (A=10% CH_3CN in buffer B=90% CH_3CN in buffer) were used to separate the individual components.

ASSAYS FOR CRF ACTIVITY. The stimulation of the release of immunoreactive ACTH in vitro was measured by three (3) procedures:

1. An isolated rat pituitary method described by Saffran and Schally (8), except that: (a) pituitary quarters and not halves were used, (b) the tissue was not cooled between incubations (9), (c) 0.25% albumin was added to Krebs-Ringer bicarbonate glucose medium (9), (d) the released ACTH was measured by RIA. The results are expressed as percentage ratio of medium ACTH in the second hour i.e. after the sample was added, to medium ACTH in the first hour. Each sample was assayed in quadruplicate.

2 and 3. Using monolayer cultures of rat (10) or mouse pituitary cells. In these cases, ACTH was expressed as pg released per ml. The concentration of ACTH in the medium was measured by a specific RIA for ACTH. The RIA was performed using a specific rabbit antiserum #851 which was generated against synthetic $ACTH^{1-24}$ adsorbed on polyvinylpyrrolidone (MW 40,000). Highly purified porcine corticotropin A (Armour) was used for both labelling with ^{125}I and the reference standard. Incubation was carried out as described by Rees et al. (11). The antiserum was used at a final dilution of x5600. The minimum detectable dose as expressed by the dose corresponding to 2 SD from the buffer control was 8 pg per tube. The standard curve was parallel to that for RIA with NIH human ACTH antiserum (Batch #2). The differences between control and experimental samples were examined by using Duncan's new multiple range test.

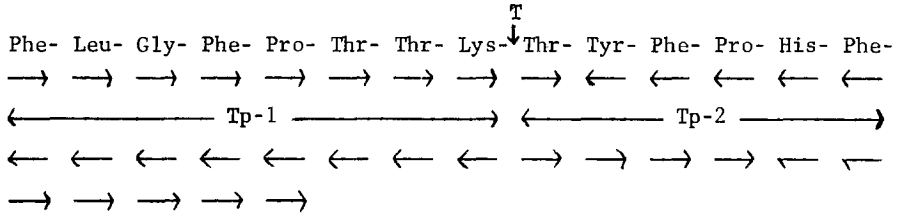
The CRF activity was also tested in vivo in rats or mice pretreated with chloropromazine, morphine and Nembutal (12), using elevation of plasma corticosterone as the index of ACTH release.

RESULTS

Isolation, Structure and Synthesis. After gel filtration of extracts (lyophilized weight=3.6 kg) from 470,000 pig hypothalami on preparative columns of Sephadex G-25 (see Fig. 1, ref. 3, 4 or 5) CRF activity as based on in vitro and in vivo assays was eluted in several fractions. In this paper we shall be concerned only with retarded, or low MW fractions with an R_f=0.39-0.27 (V_E/T=0.93-1.3, tubes 1257-1696 Fig. 1, ref. 3, 4 or 5). Some of the CRF activity of this fraction was due to catecholamines, which persisted through two more purification steps, phenol extraction (4, 5), which reduced the weight from 380g to 70g and chromatography on CMC (Fig. 2, ref. 4). Fractions No. 120-145 with a conductivity of 4m MHOS yielded 657 mg, and were purified in two batches (405mg and 192mg respectively) by CCD in a system consisting of 0.1% acetic acid: 1-butanol: pyridine=11:5:3, exactly as reported in Fig. 3 ref. 4. The CCD effected a complete separation of

FIGURE 1

Summary of the Sequence Studies on the Tetradecapeptide



Residues identified by:

⟶ amino acid analysis after back hydrolysis of PTH derivative and two dimensional TLC of DNS-amino acids.

⟵ Carboxypeptidase Y digestion ⟵ Carboxypeptidase A digestion

T ↓ Site of Cleavage with Trypsin

Tp-1 and Tp-2: Tryptic fragments

catecholamines (K=0.6) from the other material with CRF activity (mean K=5). Batch I, yield 37mg, was repurified on Sephadex G-15 using M acetic acid as the eluant (R_f=0.62, yield 10.3mg) and then by partition chromatography as in Fig. 5 ref. 3. The yield of this material (R_f=0.63), designated preparation I, was 5.6mg. Batch II from CCD was repurified by chromatography on SE-Sephadex, using pyridine acetate buffers. A symmetical peak which emerged with a conductivity of 9.3m MHOS yielded 4.5mg of preparation II after lyophilization. Both preparations were homogeneous by TLC in the systems described previously (3, 4). Amino acid analysis (3-6) showed the composition: $Thr_3Pro_2Gly_1Leu_1Tyr_1Phe_4His_1Lys_1$. The amino acid content was 61% of dry weight. The structural approach included the Edman-dansyl degradation (13) of intact peptide and its tryptic digest as well as carboxypeptidase A and Y digestion (Fig. 1). The two tryptic fragments Tp-1 and Tp-2 were separated by HPLC. On the basis of these results (Fig. 1), the structure was determined to be Phe-Leu-Gly-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe which, it was then discovered, represents residues 33-46 of porcine hemoglobin α-chain (14, 15). This tetradecapeptide was then synthesized by solid

TABLE I

EFFECT OF NATURAL AND SYNTHETIC TETRADECAPEPTIDE ON THE RELEASE OF ACTH FROM ISOLATED RAT PITUITARY QUARTERS

EXP	MATERIAL	DOSE μg/ml	Medium ACTH % of control, 2nd incubation/1st incubation x 100	P vs Control
I	Control	--	46.2 ± 5.3	--
	Natural Prep I	1.0	80.9 ± 4.9	< 0.01
II	Control	--	95.4 ± 10.2	--
	Natural Prep I	3	155.8 ± 7.3	< 0.01
III	Contral	--	78.0 ± 6.1	--
	Natural Prep II	2.5	168.3 ± 3.4	< 0.05
IV	Control	--	71.7 ± 7.9	--
	Natural Prep II	4.5	148.9 ± 18.9	< 0.01
V	Control	--	68.4 ± 5.6	--
	Natural Prep II	1.5	99.0 ± 2.5	< 0.01
		4.5	115.3 ± 7.1	< 0.01
VI	Control	--	76.4 ± 15.2	--
	Natural Prep II	1.5	130.5 ± 8.8	< 0.05
	Synthetic Prep	1.5	122.0 ± 11.9	< 0.05
VII	Control	--	75.2 ± 7.5	--
	Synthetic Prep	0.75	91.6 ± 3.9	NS
		1.5	136.9 ± 8.7	< 0.05

phase methods. The R_f's of natural and synthetic material and their tryptic digestion products were identical in several solvent systems.

BIOLOGICAL CHARACTERIZATION

Both preparations of the natural material and the synthetic tetradecapeptide stimulated the release of ACTH from isolated rat pituitary quarters in doses of 1.0-4.5 μg/ml (Table I). The synthetic tetradecapeptide appeared to cause the liberation of similar amounts of ACTH in this system as the natural materials, but there was no clear cut dose-response relationship and occasionally doses larger than 3-4.5 μg/ml appeared to inhibit ACTH release. The tetradecapeptide itself did not show any ACTH immunoreactivity. When monolayer cultures of mouse

TABLE II

EFFECT OF NATURAL AND SYNTHETIC TETRADECAPEPTIDE ON THE RELEASE OF ACTH FROM MONOLAYER CULTURES OF MOUSE ANTERIOR PITUITARY CELLS

EXP	MATERIAL	DOSE μg/ml	pg/ml ACTH ± SE	P vs Control
1	Control	--	2225 ± 28	--
	Natural Prep I	0.025	2956 ± 183	< 0.05
		0.10	3764 ± 129	< 0.01
		0.40	4189 ± 452	< 0.01
2	Control	--	6190 ± 74	--
	Natural Prep II	0.1	8319 ± 248	< 0.01
		0.4	9704 ± 364	< 0.01
		1.6	12238 ± 851	< 0.01
		6.4	13031 ± 351	< 0.01
3	Control	--	1937 ± 135	--
	Natural Prep I	0.5	2995 ± 187	< 0.01
	Synthetic Prep	0.5	3154 ± 201	< 0.01
4	Control	--	3873 ± 201	--
	Synthetic Prep	0.025	4615 ± 250	< 0.01
		0.10	4822 ± 70	< 0.01
		0.40	5290 ± 42	< 0.01
5	Control	--	864 ± 41	--
	Synthetic Prep	0.025	990 ± 30	NS
		0.10	1179 ± 69	< 0.05
		0.40	1547 ± 167	< 0.01
		1.60	2367 ± 120	< 0.01
		6.40	3193 ± 213	< 0.01

pituitary cells were used, there was, in most cases, a significant dose-response relationship between 0.1 and 6.4 μg/ml (Table II). In a time-response experiment, 5 μg/ml of this tetradecapeptide increased ACTH release from mouse monolayer cultures within 20 min and maximum levels were reached at 4 hr. Incubation of the synthetic tetradecapeptide with rat plasma for 15 min at 37° abolished its CRF activity. Preincubation of mouse pituitary cells with 1-10 μg/ml of dexamethasone inhibited the rise in ACTH in response to the tetradecapeptide, but dibenzyline, in doses of 25 μg/ml, an adrenergic blocker, did not alter ACTH release.

When monolayer cultures of rat pituitary cells were used, natural preparations of the tetradecapeptide occasionally showed some CRF activity in doses of

1 μg/ml. Analogs of this tetradecapeptide having the C-terminal sequence -Pro-Phe-His-Phe-OH and -Pro-His-Phe-Phe-OH were also active in vitro. However, the natural and the synthetic tetradecapeptide were inactive in vivo in mice and rats pretreated with chloropromazine-morphine and Nembutal, when injected intravenously in doses from 5-200 μg.

DISCUSSION

Although this appears to be the first peptide isolated from porcine hypothalami on the basis of the ability to release immunoreactive ACTH in vitro, it is obviously an artefact. It was probably formed from α-chain of hemoglobin during the dissection from pig brains or extraction procedure with 2 N acetic acid, in spite of the fact the extracts were kept at 8°C and then boiled to inactivate the proteolytic enzymes. The enzyme responsible could be possibly cathepsin D. This tetradecapeptide is unlikely to have an origin independent from that of hemoglobin, its sequence corresponding to residues no. 33-46 of the α-chain, as determined by Braunitzer et al. (14). The residue no. 37 in the α-chain (and no. 5 in this tetradecapeptide) is Pro, as correctly deduced by Braunitzer (14) and not Thr as suggested by Yamaguchi (15). Because of its origin and because it is inactive in vivo and does not show a dose-response relationship in vitro using pituitary quarters, this tetradecapeptide is unlikely to be a physiological corticotropin releasing hormone (CRH). It is, nevertheless, interesting that this peptide can release ACTH in several in vitro assay systems, although activity is much smaller than that of other CRF preparations which are effective at 3 ng/ml (1). The multiplicity of substances with CRF activity was proposed by us more than 17 years ago (17) and our recent work (1, 2) and that of others (18, 19) confirms our early findings. This multiplicity of CRF is difficult to interpret at present, but in the search for the physiological CRF, caution must be exercised in interpreting the results based solely on stimulation of ACTH release in vitro.

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