Isolation and amino acid sequence of corticotropin-releasing factor from pig hypothalami

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Abstract

A polypeptide was isolated from acid extracts of porcine hypothalami on the basis of its high ability to stimulate the release of corticotropin from superfused rat pituitary cells. After an initial separation by gel filtration on Sephadex G-25, further purification was carried out by reversed-phase HPLC. The isolated material was homogeneous chromatographically and by N-terminal sequencing. Based on automated gas-phase sequencing of the intact and CNBr-cleaved peptide and on carboxypeptidase Y digestion, the primary structure of this 41-residue polypeptide was determined to be $\text{Ser-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Met-Glu-Asn-Phe-NH}_3$. Porcine corticotropin-releasing factor (CRF) shares a common amino acid sequence (residues 1–39) with rat and human CRF and differs from these only in positions 40 and 41. However, isoleucine was also present at position 40 in porcine CRF, but in a smaller percentage than asparagine. The sequence of porcine CRF shows 83% homology with ovine CRF. Porcine CRF markedly stimulated the release of corticotropin from superfused rat and pig pituitary cells. The biological activity and close structural relationship to CRF's of other species indicate that the peptide isolated represents porcine CRF.

MATERIALS AND METHODS

Purification. A total of 470,000 pig hypothalami were dissected, defatted, and extracted with 2 M acetic acid as reported (4, 7). The extracts (lyophilized weight, 3.6 kg) were purified by preparative gel filtration on Sephadex G-25 (7, 20). After gel filtration, CRF activity was eluted in several fractions. CRF activity in retarded fractions has been described (20). This paper reports the characterization of high molecular weight fractions with CRF activity, which emerged near the void volume. Ten-gram amounts of these Sephadex fractions (nos. 557–656, $R_f = 0.82–0.7$; see fig. 1 of ref. 20) representing an equivalent of 16,600 hypothalami were dissolved in 1 liter of solvent A (0.1% trifluoroacetic acid) containing 0.2% 2-mercaptoethanol and centrifuged at 17,500 x g for 20 min. The supernatant was pumped onto a 2.5 x 50 cm glass column, packed with SynChroprep RP-P (Syn Chrom, Linden, IN), and preequilibrated with 2 liters of solvent A. This step was repeated in 10 separate runs; thus a total of 100 g, corresponding to 166,000 hypothalami, was purified.

For both the semipreparative and analytical separations, prepacked Vydis 218 TP5 (5-μm particles) reversed-phase columns were used. In the first three purification steps (Table 1), the samples were pumped onto preequilibrated columns, but in the last four stages the materials were injected through a Beckman model 210 injector. For the trifluoroacetic acid runs, solvent B was acetonitrile/water (60:40) containing 0.1% trifluoroacetic acid. For the heptfluorobutyric acid separations solvent A consisted of 0.1% heptfluorobutyric acid in water and solvent B was acetonitrile/water (60:40) with 0.1% heptfluorobutyric acid. The fractions were collected in siliconized (AquaSil, Pierce) glass tubes containing 2-mercaptoethanol (0.2% of fraction volume). The HPLC system used throughout the purification procedure was a Beckman gradient liquid chromatography system consisting of two 114 M pumps, an M 420 system controller, and an M 160 UV detector or a Uvicord S monitor (LKB).

CRF Assays. The fractions collected in steps 1–3 (Table 1) were tested by RIA for CRF content and immunoreactive regions were subjected to a bioassay (21). Further purification was monitored only by the bioassay, because the RIA procedure (22), using anti-ovine CRF serum was not specific enough for quantifying the CRF activity. The bioassay was based on the stimulation of the release of corticotropin in vitro from rat anterior pituitary cells superfused on a column (21). Twelve female Sprague–Dawley rats were used in each experiment. For testing the peptides at the final purification stage, a 10-kg pig was also used. The anterior pituitaries were removed and treated with collagenase, dispersed, and transferred into two chambers of the superfusion apparatus as described (21). The cells were superfused overnight with medium 199 (Sigma) at a flow rate of 20 ml/hr. The samples were administered every half hour for 3 min and 1-ml fractions were collected at 3-min intervals. Ovine CRF at 0.2

Abbreviation: CRF, corticotropin-releasing factor.

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Contributed by Andrew V. Schally, August 25, 1985
and 2 nM was used as the standard. The corticotropin released into the medium was measured by a specific RIA for corticotropin (21, 22).

**Amino Acid Analyses.** Samples were hydrolyzed in 5.7 N HCl for 22 hr at 110°C and analyzed on a Liquimat III amino acid analyzer (23).

**Cyanogen Bromide Cleavage.** Porcine CRF (200 pmol) was incubated with CNBr in 150 µL of 70% formic acid for 23 hr at room temperature in the dark, using a 200-fold molar excess of CNBr over the methionine content in the sample. The volume was then reduced to 60 µL under a stream of N₂ and applied directly onto the glass filter of the gas-phase sequencer (24).

**Automated N-Terminal Sequencing.** Intact porcine CRF and fragments of porcine CRF produced by CNBr cleavage were subjected to gas-phase sequencing with a 470A Applied Biosystems gas-phase sequencer employing Polybrene as carrier and a standard single-coupling, single-cleavage program. The resulting phenylthiohydantoin derivatives of amino acids were identified using a Hewlett-Packard 1084B HPLC (25).

**C-Terminal Analyses Using Carboxypeptidases A and Y.** Porcine CRF (250 pmol) was dissolved in 30 µL of 0.1 M Na₂HPO₄ (pH 5.3), and carboxypeptidase Y was added in 1.5 µL of H₂O, at a 1:50 enzyme-to-substrate ratio. After digestion for 15 min at 35°C, 10 µL of glacial acetic acid was added and the sample was lyophilized, dissolved in 0.2 N sodium citrate (pH 2.2), and subjected to amino acid analysis. An enzyme blank was also run. Carboxypeptidase A digestion of 250 pmol of porcine CRF was carried out at 37°C in 0.05 M NH₄HCO₃ at a 1:50 enzyme-to-substrate ratio. After 30 min, 10 µL of glacial acetic acid was added and the sample was subjected to amino acid analysis.

**RESULTS**

The isolation of peptides with CRF activity was carried out using more strongly retained fractions obtained during preparative-scale HPLC purification of growth hormone-releasing hormone (GH-RH) (Table 1, step 1). GH-RH was eluted earlier and was well-separated from CRF fractions, which emerged much later. The dry weight of combined fractions with CRF activity from 10 such runs was 6.5 g. In step 2 (Table 1), this material was dissolved in 800 ml of 0.1% trifluoroacetic acid and the solution was pumped onto a pre-equilibrated SynChroprep RP-P column. The elution was performed using a less steep gradient than that in step 1 (Table 1). The pooled lyophilized fractions with CRF activity (0.74 g) were dissolved in 100 ml of solvent A and pumped onto two equilibrated semipreparative Vydac columns connected in series. The elution was carried out as indicated for step 3 in Table 1. This procedure resulted in two overlapping CRF-active fractions. The zone that was eluted earlier had a higher CRF activity. The CRF activity in the fraction that emerged later was much lower and in the subsequent HPLC steps, these two CRF components were purified separately. To avoid major losses, pooled fractions of active zones were not lyophilized after step 3 but were injected in increments at 2-min intervals (Table 1) while solvent A was pumped through the column. The readsorption on the column was the result of an "in situ" reduction in the acetoniitride content of the sample. In step 4, the fraction with major CRF activity was rechromatographed in a semipreparative heptfluorobutyric acid system (Table 1). Although the CRF activity was eluted as a sharp zone, indicating a high degree of purification, rechromatography of this fraction in a semipreparative trifluoroacetic acid system (step 5) separated it into three overlapping CRF-active regions (fractions A–C, Fig. 1).
Fractions A and B showed high activity and were purified separately. Table 1 shows the HPLC conditions for steps 6–8, aimed at the purification of peptides in fraction A. In step 6, fraction A was further divided into two active components, A1 and A2 (Fig. 2). The major and more active component (A2) was rechromatographed in step 7, yielding two partially resolved peaks (A21 and A22) of highly active material. A22 was rechromatographed and concentrated in step 8 (Fig. 3). Fraction A22 was highly active in stimulating ACTH release from rat and pig anterior pituitary cells superfused on a column (Fig. 4). A22 appeared to be the main peptide with CRF activity on the basis of the yield (18 μg) and its high potency. Aliquots of fraction A22 were subjected to amino acid and structural analyses. Other peptides with CRF activity were purified by a scheme similar to that shown in Table 1. Their characterization will be reported separately.

The complete amino acid sequence of porcine CRF (Fig. 5) was determined by automated N-terminal sequencing of the intact porcine CRF (A22) and of peptides produced by cleavage of A22 with CNBr (without separation) and by carboxypeptidase Y digestion of the intact peptide. Amino acid composition of intact porcine CRF confirmed these results. Automated gas-phase sequencing of A22 elucidated the first 38 of 41 amino acids in porcine CRF. The yields, in pmol, of phenylthiohydantoin derivatives of amino acids were as follows: (1) Ser, 87; (2) Glu, 420; (3) Glu, 440; (4) Pro, 427; (5) Pro, 460; (6) Ile, 380; (7) Ser, 67; (8) Leu, 350; (9) Asp, 321; (10) Leu, 330; (11) Thr, 58; (12) Phe, 218; (13) His, 103; (14) Leu, 215; (15) Leu, 227; (16) Arg, 135; (17) Glu, 170; (18) Val, 185; (19) Leu, 175; (20) Glu, 155; (21) Met, 148; (22) Ala, 153; (23) Arg, 81; (24) Ala, 139; (25) Glu, 111; (26) Gin, 105; (27) Leu, 108; (28) Ala, 93; (29) Gin, 88; (30) Gin, 93; (31) Ala, 75; (32) His, 51; (33) Ser, 28; (34) Asn, 50; (35) Arg, 31; (36) Lys, 57; (37) Leu, 44; (38) Met, 37. The average yield per cycle was 93.7%. Methionine residues were found in positions 21 and 38. Since the sequencing of porcine CRF was completed through residue 38 and the sequences of two of the three peptides produced by CNBr cleavage were known, the sequence of the third (and C-terminal) peptide could be deduced by sequencing the CNBr-generated peptides, without separating them (8). This analysis revealed that the C-terminal peptide sequence was Glu-Asn-Phe-NH₂. However, there was a microheterogeneity at the penultimate C-terminal position; isoleucine was present at a ratio of about 1:2 as compared to asparagine. This microheterogeneity could arise from the presence of different strains among the large number of pigs (470,000) used as source of material for these structural studies.

Carboxypeptidase Y digestion of intact porcine CRF (A22) resulted in the liberation of phenylalaninamide, isoleucine, and asparagine in a molar ratio of 1:0.3:0.5. Conversely, digestion of intact CRF (A22) with carboxypeptidase A did not release any free amino acids. These results confirm the structural conclusions based on the sequencing of peptides obtained by CNBr cleavage of A22.

**DISCUSSION**

An efficient HPLC strategy was developed for the isolation of peptides with CRF activity present in minute quantities in hypothalamic extracts. Our purification approach made use of the latest developments in the fields of reversed-phase HPLC packing materials and solvent systems (26–28). In our purification process some essential features of displacement chromatography (29) were also used. The amount of peptides loaded onto the column was 8–15 times greater than the normal maximal load on columns of comparable bed volume used in elution-mode chromatography (30). To reduce losses in the last, critical steps, we tried several methods unsuccessfully (31). However, our incremental-injection method consistently yielded >80% recovery, even when 1 or 2 μg of...
peptide in a volume of about 10 ml had to be rechromatographed.

The structure of porcine CRF shows a greater resemblance to rat and human CRF, the sequences of which are identical (15–17), than to ovine CRF and caprine (11, 12, 32) or bovine CRF (33). Porcine CRF shares the common N-terminal 39 amino acid sequence with rat/human CRF but differs from rat/human CRF in positions 40 and 41, which are occupied by (Asn/Ile)-Phe in porcine CRF and by Ile-Ile in rat/human CRF (15, 16). The change from Asn (porcine) to Ile (other species) in position 40 and from Phe (porcine) to Ile (rat/human) in position 41 of CRF requires only a single base change in the codons for these amino acids. However, the change from Ala (ovine/caprine, bovine) to Ile (rat/human) or to Phe (porcine) at position 41 represents a 2-base change in these codons. In addition to these changes in the C-terminal sequence, porcine, rat, and human CRF differ from ovine and caprine CRF at positions 2, 22, 23, 25, 38, and 39 and from bovine CRF at position 33 as well. Porcine CRF contains regions homologous with peptides such as sauva-gine, derived from frog skin, and urotensin I, isolated from the urophysis of the teleost fish catostomus (34, 35). These two peptides exhibit CRF activity (11, 34, 35).

Various studies have established that CRF plays a physiological role in the response to stress. Brown et al. (36) showed that CRF injected into the brains of rats stimulates sympathetic outflow and produces hyperglycemia and an increase in plasma concentrations of glucagon, epinephrine, and norepinephrine. Ovine CRF is active in rats (11, 37), sheep (38), monkeys (39), and humans (40, 41) and raises not only the plasma corticotropin concentration but also β-lipotropin and β-endorphin levels. An elevation in plasma levels of corticotropin in rats and of cortisol in sheep, monkeys, and humans has also been demonstrated after CRF administration (37–41). The response to CRF in these species is blocked by dexamethasone. Similarly, treatment with anti-CRF antiserum blocks the corticotropin release that follows various types of stress (42).

CRF has been localized immunocytochemically in the hypothalamus of rats, guinea pigs, sheep, and other species (43, 44). This suggests that CRF could be released from the nerve endings in the median eminence into the hypophyseal portal vessels and transported to the pituitary, where it would activate the corticotrophs to release corticotropin. CRF receptors are present in the pituitary (45). CRF has been found in the hypophyseal portal blood of rats (46). Hemorrhage-induced secretion of CRF into hypophyseal portal circulation can be inhibited by pretreatment with glucocorticoids (47).

Because the physiological role of CRF in the response to stress has been documented by various studies (11, 36–39, 42, 46, 47) and the structures of CRF from various species have been determined (11, 15–17, 32, 33), its name could be changed from “factor” to “hormone.” This is in accord with original proposals for hypothalamic substances that have had their structures determined and that have been shown to be the likely physiological regulators of the secretion of the respective anterior pituitary hormones (48). The isolation and structural characterization of porcine CRF, reported here, represents for one of us (A.V.S.) the culmination of work started more than 25 years ago.

The participation, in various earlier phases of this project, of Drs. R. C. C. Chang, W.-Y. Huang, S. Vigh, and L. Torres-Aleman and many others is gratefully acknowledged. A special mention is due to Dr. Murray Saffran for joint entry into the CRF field some 30 years ago. We thank the National Hormone and Pituitary Program for gifts of materials used in RIAs. This work was supported by National Institutes of Health Grant AM07467 and the Veterans’ Administration Research Service.

9. Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M.,