Immunological detection of pro-corticotropin releasing factor (CRF) in rat hypothalamus and pancreatic extracts

Evidence for in vitro conversion into CRF

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Extracts of both rat hypothalamus and pancreas were analyzed for their corticotropin releasing factor (CRF)-like immunoreactivity by radioimmunoassay (RIA). In the case of the hypothalamus, besides the rat CRF, further identified by high-pressure liquid chromatography (HPLC), two peptide components, a 20-kDa and a 10-kDa species were detected. The 20-kDa component was stable under acidic pH conditions and was further purified by reverse-phase HPLC. When exposed to proteolytic activities coeluting with 'high-molecular-mass CRF' at pH 6, processing was observed and the CRF generated was identified both by RIA, molecular sieve filtration and HPLC under different experimental conditions. It is concluded that this 20-kDa CRF may represent the CRF precursor and that hypothalamic extracts may contain processing enzymes involved in its selective post-translational cleavage. In the pancreatic extract two immunoreactive forms of CRF were detected, the smaller coeluting with the rat CRF and the other corresponding to the intermediate 10-kDa component detected in the hypothalamus. Pancreatic rat CRF, analyzed using RIA both by molecular sieve filtration and HPLC, was indistinguishable from the hypothalamic rat CRF.

Corticotropin precursor *Proteolytic enzyme* *HPLC* *Corticotropin RIA*

1. INTRODUCTION

Direct evidence for an hypothalamic corticotropin (ACTH) releasing factor (CRF) that stimulates ACTH secretion from the pituitary gland was first reported in 1955 [1,2]. More than 25 years later, isolation and sequencing of a 41-amino-acid hypothalamic peptide from ovine (o-CRF) [3,4] and rat (r-CRF) [5], with corticotropin releasing activity were performed.

The available data suggest that the hypothalamic CRF enhances synthesis and secretion not only of ACTH but also of related peptides such as B-endorphin, B-lipotropin and α-melanocyte-stimulating hormone [6,7]. Moreover, it has been shown that o-CRF also stimulates somatostatin secretion from cultured brain cells [8]. Recently the primary structures of the biosynthetic precursor of both ovine CRF [9] and human CRF [10] were predicted by determining the nucleotide sequence of the cloned DNA complementary to the mRNA encoding the precursor. Indeed, a number of hormonal peptides have been detected both in the central nervous system and in the gastrointestinal tract [11,12]. This differential organ distribution may reflect the different biological role of these compounds [13]. Recently immunohistochemical studies revealed pancreatic systems that stain with antiserum to CRF [14]. Whether this material represents chemically authentic CRF was unclear.

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All these data are consistent with a multiple, extracellular, regulatory role for CRF.

However to date: (i) in the hypothalamus no direct experimental evidence for the presence of CRF precursor molecules and for their processing has been reported; (ii) no analysis and biochemical characterization of the pancreatic immunoreactive CRF component have been performed.

Here we have characterized, in the rat hypothalamus, a high-molecular-mass peptide immunologically related to CRF, that can be converted into CRF by a proteolytic activity present in the hypothalamic extract. We have also shown that the pancreatic CRF-like peptide, characterized by several methods including HPLC, is identical to hypothalamic CRF.

2. MATERIALS AND METHODS

2.1. *Extracts and fractionation*

Rat hypothalami (5 fragments) or rat pancreas (2 fragments) were removed immediately after death by rapid decapitation and homogenized in a Potter Elvejhem (clearance 25 μm) in 2 ml of 0.2 N HCl, 8 M urea. After centrifugation for 1 h at 100000 × g (Beckman L5 50, SW 50 rotor) the supernatant was submitted to gel filtration over a Sephadex G-75 column (1.6 × 90 cm) equilibrated and eluted with 0.1 N HCOOH at 4°C. Further fractionation of the different molecular mass species was performed over a Sephadex G-75 column (0.9 × 50 cm) under the same conditions as above.

2.2. *High-pressure liquid chromatography (HPLC)*

The HPLC system consisted of a Beckman apparatus (pumps A 110, directed by a 421 controller). Reverse-phase HPLC was performed using a μBondapack CN column (Waters) under the conditions described in the figure legends.

2.3. *CRF radioimmunoassay*

Radioimmunological detection of CRF was performed using an antiserum raised against o-CRF (231 06 07) at a final dilution of 1/30000 in a medium containing potassium phosphate buffer (pH 7.4), 0.25 M NaCl, 2.5 mg/ml BSA and 0.1% Triton X-100. ^{125}I-labelled Tyr o-CRF (ovine), obtained by labeling of Tyr-O-CRF (Peninsula Laboratories, San Carlos, CA) with the lactoperoxidase method, was used as marker. The antigens (either the standard peptide or a biological sample) were first preincubated 24 h with the anti-CRF immune serum, then the tracer was added and the mixture left for 18 h. The free iodinated peptide was separated from the bound peptide by the double-antibody method using anti-rabbit γg globulins and 1 ml of 6% polyethylene glycol. Under these conditions the detection limit was 2 pg/test and a 50% displacement of the tracer was obtained with 7 pg/test (1.4 fmol) of o-CRF. The cross-reaction with biosynthetic r-CRF was only 50%. In the CRF radioimmunoassay, controls were systematically performed to establish that the measured immunoreactivity was not due to either degradation of the labeled tracer or to sticking to a protein component.

3. RESULTS

Analysis of rat hypothalamic extracts by molecular sieve filtration over a Sephadex G-75 column (fig.1) resolved two major immunoreactive peaks and one minor intermediate peak. The lower M_r immunoreactive species was identified as r-CRF as it coeluted with synthetic o-CRF or r-CRF filtered under the same conditions and tested by RIA. The apparent molecular mass of the large immunoreactive form of r-CRF was 20 kDa ± 10% (calculated by comparison with the elution volume of globular proteins used for calibration). Control experiments were conducted to verify this large apparent molecular mass. The elution profile and the apparent molecular mass of the immunoreactive 20-kDa material were unmodified when reanalyzed by the same molecular sieve filtration after 2 h exposure to 8 M urea (fig.1, inset). These results indicate the presence, in rat hypothalamus extracts, of a higher M_r component immunologically related to r-CRF, and which is not an aggregate of lower size antigens. Under the same conditions, analysis of the rat pancreatic extract showed a relatively similar pattern (fig.1) except that the 20-kDa component was not detectable. This may be due to the high content of protease activity present in the pancreas as compared to the hypothalamus. However, the major immunoreactive peak detected in the pancreatic extract filtration has the same molecular mass as the hypothalamic CRF.

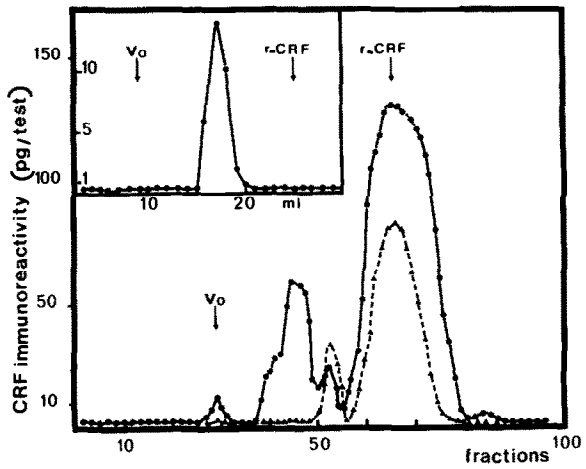

Fig.1. Molecular sieve filtration of CRF immunoreactive forms extracted from rat hypothalamus and rat pancreas. An acid/urea extract of hypothalami or pancreas was submitted to gel filtration over a Sephadex G-75 column (1.6 × 90 cm) equilibrated and eluted with 0.1 M HCOOH (10 ml/h) at 4°C. An aliquot of each fraction (1 ml) was tested for CRF immunoreactivity: hypothalamic extracts (●—●), pancreatic extracts (▲---▲). Results are expressed as pg CRF/test. The arrow indicates the elution position of synthetic rat CRF filtered under the same conditions. V_o, exclusion volume. (Inset) Analysis of the hypothalamic 20-kDa species recovered from the Sephadex G-75 gel filtration step. The high-molecular-mass immunoreactive material (fractions 40–50 of the gel filtration) was lyophilized, resuspended for 2 h in 1 ml acid/urea buffer and reanalyzed on a Sephadex G-75 column (0.9 × 50 cm at 5 ml/h). CRF immunoreactivity was tested on each fraction (1 ml), and the results expressed as above. The arrow indicates the elution position of the synthetic rat CRF.

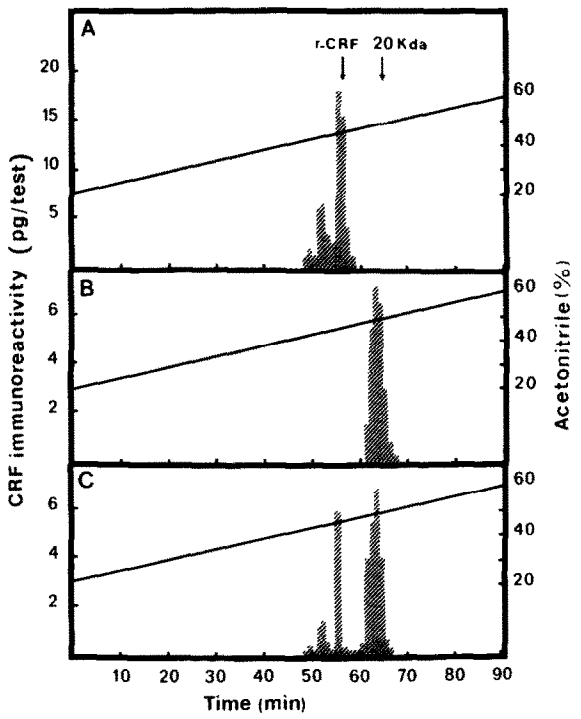

Fig.2. Processing of the purified high-molecular-mass species by hypothalamic enzyme activity. (A) Fractions corresponding to the r-CRF recovered from the Sephadex G-75 column of fig.1 were pooled, lyophilized, then redissolved in 0.5 ml of 1% TFA and applied to a μBondapack CN column (4 × 250 mm). After injection, the product was eluted with a linear gradient of acetonitrile (0.44%/min) at a flow rate of 1 ml/min over 90 min. Each fraction (1 ml) was tested for CRF immunoreactivity. (B) Chromatography of the high-molecular-mass species (20 kDa) recovered from the Sephadex G-75 column of fig.1. Same conditions as in A. (C) An aliquot of the 20-kDa material recovered from the experiment of fig.1 was incubated for 1 h at 37°C in ammonium acetate buffer (pH 6), in the presence of 100 kallikrein inhibitor units (KIU)/ml of aprotinin. After incubation, the medium was acidified with 1% TFA and submitted to a filtration on μBondapack CN column under the same conditions as in A. Results are expressed as pg CRF/test. The arrows indicate the elution position of synthetic r-CRF filtered in the same conditions, and of the 20-kDa polypeptide.

Precursor-product relationships between the 20-kDa form and hypothalamic r-CRF were assessed by in vitro conversion experiments and the products were analyzed using reverse-phase HPLC. The hypothalamic r-CRF material isolated by molecular sieve filtration (fig.1) was applied to a μBondapack CN column and eluted with a linear gradient of acetonitrile (0.44%/min) in aqueous 0.1% trifluoroacetic acid (TFA). After elution the r-CRF immunoreactivity was recovered as a major peak (retention time: 56 min) corresponding to 80% of the total immunoreactive material recovered (fig.2A). This main species coelutes with synthetic r-CRF run under the same gradient conditions. The two minor peaks (retention time 49 and 52 min, respectively) probably correspond to hypothalamic r-CRF molecules slightly modified during the extraction procedure, since they were recognized by the anti-CRF antibodies and have a

molecular mass similar to that of synthetic r-CRF by molecular sieve filtration (not shown).

Under the same gradient conditions the 20 kDa CRF-like product, first purified by gel filtration, was eluted with a retention time of 63 min (fig.2B). This higher M_r material, recovered from the experiment of fig.1, was submitted to a processing experiment. This material was first lyophilized and resuspended in 0.1 M phosphate buffer (pH 6) in the presence of 100 kallikrein inhibitor units/ml of aprotinin to avoid non-specific degradation by serine proteases present in the extract. The mixture was then incubated for 1 h at 37°C. Under these conditions specific proteolytic activity coeluting with the 20 kDa polypeptide was able to process this material. Analysis of the products thus generated by means of reverse-phase HPLC filtration showed (fig.2C) two main distinct immunoreactive forms. A noticeable fraction of uncleaved 20-kDa pro-form together with the immunoreactive species eluting with the same retention time as the hypothalamic r-CRF were observed. In addition, we also found the two minor components described above (fig.2A). Analysed by molecular mass filtration, the 20-kDa species generates an immunoreactive CRF-like peptide which coelutes with synthetic or hypothalamic r-CRF when filtered over Sephadex G-75 (not shown). Further identification of both the immunoreactive hypothalamic peptide generated by the processing experiment and the pancreatic immunoreactive CRF-like component was achieved by reverse-phase HPLC analysis under isocratic conditions. This allowed separation of the o-CRF (retention time 25 min) from the r-CRF (retention time 38 min), which exhibits 7 amino acid substitutions in comparison with the o-CRF sequence. Fig.3 shows first that the immunoreactive peptide generated by the processing experiment represented in fig.2C has the same retention time as the synthetic r-CRF, and second that when the pancreatic peptide isolated by molecular sieve filtration (fig.1) is filtered under the conditions described above, it coelutes with the synthetic r-CRF. These observations indicate (i) that the 20-kDa component may represent the biosynthetic precursor of the CRF, and (ii) that pancreatic tissues contain a peptide exhibiting an immunological and biochemical behaviour similar to the rat CRF.

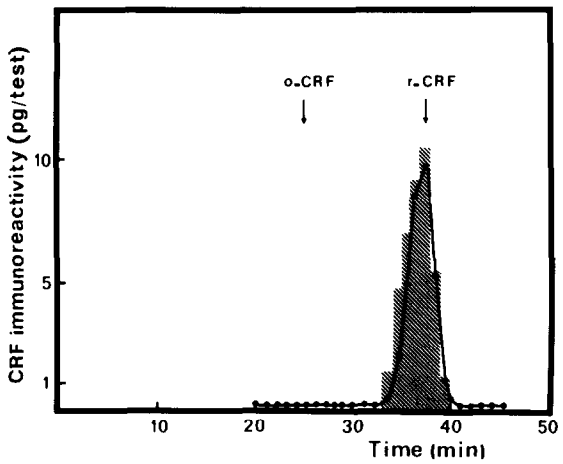

Fig.3. HPLC of the generated hypothalamic r-CRF-like peptide and of pancreatic CRF. The r-CRF immunoreactivity recovered from filtration in fig.2C was lyophilized, solubilized in 0.5 ml of 1% TFA and injected on a μBondapack CN column. Elution was performed under isocratic conditions with 35% acetonitrile in aqueous 0.1% TFA (v/v), at a flow rate of 1 ml/min over 50 min (hatched bars). The pancreatic r-CRF material recovered from Sephadex filtration (fig.1) was filtered through the μBondapack column under the same conditions as above (●——●); each fraction (1 ml) was tested for CRF immunoreactivity. The arrows indicate the elution position of both synthetic o-CRF and synthetic r-CRF, filtered under the same conditions.

4. DISCUSSION

The present data provide immunological and biochemical evidence for the presence of a 20-kDa (± 2 kDa) immunoreactive form of CRF in rat hypothalamus extracts. This value is close to those of the ovine or human pre-pro-CRF molecules, calculated from their primary structure (minus the signal peptide sequence) as deduced from the corresponding cDNA or gene sequences [9,10]. The demonstration that this 20-kDa hypothalamic CRF can generate the r-CRF peptide by proteolytic cleavage suggests that it can be considered as a biosynthetic precursor of this hormone (fig.3). In the pancreas extract, the high content of protease activity present in this organ might explain the inability to detect this 20-kDa species. The minor intermediate-size immunoreactive components (10 kDa) also observed by molecular sieve filtration of both hypothalamic and pancreatic extracts

(fig.1, fractions 50–55) may represent intermediates in the post-translational events leading from the larger precursor to the 'final' secretory product(s). Analyzed by reverse-phase HPLC, this minor product eluted as a single immunoreactive peak with a retention time between those of the 20-kDa species and r-CRF (not shown).

Apart from its role as hypothalamic releasing factor, CRF appears to be a typical 'gut-brain' peptide, localized both in neurones throughout the brain and in the pancreas. The functional significance of this finding is as yet unknown. However, it has been shown that CRF (as well as the related peptides sauvagine and urotensin I) possesses vasomotor activity [15,16]. Thus, pancreatic CRF may play a hormonal role in the local regulation of blood flow through the endocrine or exocrine pancreas. Whether this CRF is synthesized by the pancreatic tissue or by exogenous neural cells innervating the pancreas remains to be established.

Examination of the sequences of both human CRF and of the corresponding precursor [10] shows the presence of basic amino acid doublets in the C-terminal portion of the CRF molecule and in the pro-sequence preceding the CRF peptide in the precursor. Assuming that these doublets or double pairs of basic amino acids constitute the recognition sites for selective endopeptidases [17], a number of fragments can be expected from post-translational processing of the pro-CRF molecule. Furthermore, an internal peptide can also be expected to be released by cleavage at position 35 of the CRF-41 [5]. Therefore, the pleiotropic actions exhibited by this peptide in the central nervous system or in the pancres [14,18,19] might reflect a differential processing of the precursor and hormone.

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