The distribution of corticotrophin-releasing factor immunoreactivity in various ovine tissues

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Abstract. The presence of CRF in various ovine tissues has been quantified by a radioimmunoassay using an antibody raised against ovine CRF synthesized in vitro. Acetic acid extracts of ovine hypothalamus, cerebral cortex, cerebellum, pituitary and a number of visceral organs assayed in serial dilutions gave inhibition curves parallel to those of synthetic CRF. The highest concentration of CRF immunoreactivity (CRF-I) was found in the pituitary (84.6 ± 8.77 ng/g wet weight) and the second highest in the hypothalamus (79.1 ± 2.77 ng/g wet weight). CRF-I in the cerebral cortex and visceral organs (stomach, duodenum, jejunum, ileum, pancreas and liver) was about 60 and 30% of that in the hypothalamus, respectively. Chromatography of extracted CRF-I from these tissues on Bio-Gel P-10, equilibrated and eluted with 0.1 M acetic acid, revealed a single immunoreactive peak corresponding to synthetic ovine CRF. These findings indicate that CRF is generally distributed throughout ovine tissues including visceral organs, although its function remains to be clarified.

The corticotrophin-releasing factor (CRF) has recently been isolated from ovine hypothalamic extracts and its primary structure determined (Vale et al. 1981; Spiess et al. 1981). The in vitro synthesis of the hormone has facilitated the application of immunocytochemistry and radioimmunoassay to study the tissue distribution of CRF (Vigh et al. 1982; Hashimoto et al. 1982). Using immunocytochemical techniques, CRF immunoreactivity has been demonstrated in the human gastro-intestinal tract as well as in the hypothalamus (Kruseman et al. 1982). However, the presence of CRF in the alimentary tract is still controversial (Blank et al. 1983). To clarify this point we have quantified CRF immunoreactivity by radioimmunoassay in various ovine tissues. In addition, the molecular size of the CRF immunoreactive peptide from these tissues was compared with CRF synthesized in vitro and found to be similar.

Materials and Methods

Materials

CRF and its analogues were synthesized by the solid-phase method (Ohashi et al. 1983). The peptide hormones used for specificity study were obtained as follows: ovine CRF 22–41 and CRF 37–41 from Dr. D.H. Coy, Department of Medicine, Tulane University, New Orleans, LA (USA), arginine vasopressin from Park Davis (USA), porcine insulin from Lilly (USA), glucagon from Sigma (USA), cyclic somatostatin from Clin-Midy (France) and ACTH1–24 from N.V. Organon (The Netherlands).

Immunoassays

Preparation of [125I]CRF. Ovine CRF (oCRF) was iodinated by the chloramine-T technique (Greenwood et al. 1963). oCRF (20 µg) was mixed with 20 µl 0.5 M PO₄
buffer, pH 7.5, 10 µl Na[125I] (1 mCi) and 20 µg chloramidine-T (in 20 µl 0.05 M PO₄, pH 7.5). After agitation for 30 sec, the peptide iodination was interrupted by the addition of sodium metabisulfite (90 µg in 20 µl of 0.05 M PO₄, pH 7.5). The iodination mixture was fractionated on a Sephadex G-75 column (0.9 x 25 cm) eluted with 0.1 M acetic acid in 0.1% bovine serum albumin. A small peak obtained just before the large peak of radioactivity was confirmed to be iodinated peptide by its immunoreactivity.

Assay procedure. A 0.05 M sodium phosphate buffer, pH 7.5, containing 0.1% bovine serum albumin, 0.25% disodium ethylenediamine tetraacetic acid (EDTA) and 100 kallikrein inhibitor units (KIU)/ml of aprotinin (Trasylol®) were used in the assay system. One hundred µl of rabbit anti-oCRF serum (1:10 000 dilution), 100 µl of either a reference standard or unknown sample, and 100 µl of [125I]CRF (ca. 8000 CPM) were incubated with 400 µl of the buffer for 48 h at 4°C. Separation of antibody with 500 µl of 0.05% charcoal (Norit A) coated with 0.25% dextran T-70 in 0.05 M sodium phosphate buffer, pH 7.5. One hundred µl of normal sheep serum (GIBCO) was added to all tubes not containing plasma. After incubation for 40 min at 4°C, the samples were centrifuged at 3000 r.p.m. for 15 min at 4°C and the charcoal-adsorbed [125I]CRF was counted in the autogamma spectrometer.

Sensitivity and reproducibility of the assay. Fig. 1 depicts a typical standard curve obtained with a 1:70 000 final dilution of the antiserum. Analysis of the standard curves revealed the minimal sensitivity of the assay, i.e., the lowest concentration of CRF that could be distinguished from zero with 95% confidence, to be 50 pg/tube. The coefficient of variation within an assay and between assay was 7.6% (N = 10) and 12.2% (N = 8), respectively.

Hormonal specificity. The specificity of anti CRF antiserum was tested using oCRF analogues, oCRF fragments and other peptide hormones (Fig. 1). The extent of cross-reactivity was almost 100% for [Nva21]oCRF and [Met(o)21]oCRF. On a weight basis the cross-reactivity of the antiserum with oCRF 22-41 and oCRF 37-41 was about 50% and less than 5%, respectively. There was no apparent inhibition of binding by arginine vasopressin, glucagon, somatostatin, AGTH1-24 and insulin (within a range of 100 pg – 1 µg per tube).

Extraction of CRF from tissues

Five male sheep weighing about 50 kg were sacrificed by exsangainuation under ether anaesthesia and portions of the brain, pituitary gland, 4th-stomach, antrum, duodenum, proximal jejunum, ileum, adrenal glands, liver and pancreas were removed and frozen on dry ice. These tissues were placed into 5 vol per weight of boiling 2.0 M acetic acid for 10 min. The cooled tissues were homogenized by a Polytron (Brinkman) and extracted peptides were recovered by centrifugation at 1000 x g for 15 min. After the pH was neutralized with 10 N NaOH, the extract was centrifuged at 100 000 x g for 30 min. The resultant supernatant was stored frozen prior to assay.

Fig. 1.
Standard curves for the CRF radioimmunoassay.
The hypothalamus from the ovine hypothalamus and duodenum were lyophilized and dissolved in 0.1 M acetic acid and were chromatographed on Bio-Gel P-10 (Bio-Rad, USA) column (2.4 x 67 cm) equilibrated with 0.1 M acetic acid at room temperature. Each 3 ml of eluent by 0.1 M acetic acid was lyophilized and dissolved with 1 ml of the assay diluent. Recovery of synthetic CRF was more than 90% under these conditions.

Table 1 shows the CRF-I in the precipitate after centrifugation at 100 000 x g for 30 min. The proportion varied somewhat from tissue to tissue: with a maximum of 25% of total CRF-I (supernatant CRF-I plus precipitate CRF) for the cerebrum and a minimum of 13% for the duodenum. The average was 19%.

The stability of CRF in the extract was also assessed. The concentrations of CRF-I in the tissue extracts did not change with storage at -40°C over several weeks. No significant loss of CRF-I was detected after incubation of synthetic CRF in the extracts from ovine hypothalamus and cerebrum for 2 h at 37°C (Table 2). Table 2 also demonstrates that CRF is relatively stable in sheep serum.

CRF-I in ovine organs
CRF-I was detected not only in the hypothalamus but also in extra-hypothalamic brain and visceral organs. The dilution curve for each organ extract

Results

Extraction of CRF from tissues
The efficiency of recovery for extraction of CRF from ovine tissues was assessed as follows. The organ portion was supplemented with synthetic CRF and the efficiency of recovery measured. In hypothalamus the recovery was 84.2%, in pancreas 75.2% and in duodenum 81.6%.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Supernatant after 1000 x g for 15 min</th>
<th>Supernatant after 100 000 x g for 30 min</th>
<th>Precipitate after* 100 000 x g for 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamus</td>
<td>13.6 ± 0.8</td>
<td>11.7 ± 0.9</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>11.6 ± 1.4</td>
<td>9.7 ± 0.9</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>Duodenum</td>
<td>4.3 ± 0.3</td>
<td>3.3 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>8.8 ± 0.6</td>
<td>5.3 ± 0.7</td>
<td>1.7 ± 0.7</td>
</tr>
</tbody>
</table>

* The precipitate was suspended with the same volume of the assay diluent as the supernatant.

Table 2

<table>
<thead>
<tr>
<th>Incubation periods at 37°C (min)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamus (12.3 ± 1.5)</td>
<td>25.0 ± 0.6</td>
<td>22.9 ± 0.6</td>
<td>25.0 ± 1.1</td>
<td>24.3 ± 0.4</td>
<td>22.7 ± 0.5</td>
</tr>
<tr>
<td>Cerebrum (9.0 ± 1.3)</td>
<td>29.5 ± 1.1</td>
<td>25.0 ± 1.1</td>
<td>24.0 ± 0.9</td>
<td>22.5 ± 0.7</td>
<td>26.0 ± 0.9</td>
</tr>
<tr>
<td>Serum (ND)</td>
<td>15.5 ± 1.0</td>
<td>14.0 ± 0.4</td>
<td>14.2 ± 0.6</td>
<td>12.4 ± 0.8</td>
<td>12.6 ± 0.7</td>
</tr>
</tbody>
</table>

Synthetic CRF (15 ng/ml) was added to each tissue extract. The value in parenthesis expresses the content of CRF-I in each tissue extract. ND: not detected.
was parallel to the standard displacement curve (Fig. 2). The highest concentrations of CRF-I were detected in the pituitary and the hypothalamus (Table 3). CRF-I in the cerebral cortex was approximately 60% of that found in the hypothalamus. CRF-I concentrations in the gastro-intestinal tract, pancreas and liver were almost the same, approximately 30% of those in the hypothalamus. A small amount of CRF-I was also found in the adrenal glands. No CRF-I was detected in untreated serum.

**Gel chromatography**

In order to characterize further the CRF-I of the tissue extracts, extracts of ovine cerebral cortex and upper jejunum were fractionated on a Bio-Gel P-10 column equilibrated with 0.1 M acetic acid. Synthetic oCRF, porcine insulin and glucagon were chromatographed under the same conditions for comparison. Both the cerebral and jejunum extracts showed a sharp peak of CRF-I at the same elution volume as synthetic CRF (Fig. 3). The recovery of CRF-I was more than 75%. There was no CRF-I in the void volume fraction.

**Discussion**

The immunoassay method described provides sufficient precision and specificity to be used for determination of CRF content in ovine tissues, although more sensitive methods have been reported (Hashimoto et al. 1982; Daniel & Vale 1982; Suda et al. 1983). No cross-reactivity with brain,
pituitary and gut peptides was detected, further indicating the specificity of the antiserum used. Met\textsuperscript{21} of oCRF is rather easily oxidized and could in principle decrease the sensitivity of our assay. However, this was found not to be the case and the antiserum reacted equally well with both the oxidized and native form.

CRF is stable in tissue extracts throughout the extraction procedure. Taking account of the recovery of synthetic oCRF by the extraction procedure (about 80%) and loss of it to the membrane fraction (an average of 19%), the efficiency of extraction is calculated as 65%. Thus, tissue contents of CRF will be underestimated by our extraction. However, the observed CRF-I in the precipitate in Table 1 might be due to non-specific binding of the antiserum with the precipitate.

CRF-I was detected in all the organs examined including the brain, pituitary, alimentary tract, pancreas, liver and adrenal. The proportionality of the dilution slopes of CRF-I in the extracts and their parallelism to that of synthetic CRF suggest antigenic similarity between CRF synthesized in tissue and ovine CRF synthesized in vitro.

It is possible that the immunoreactivity is due either to a biosynthetic variant of CRF such as the prohormone or a different cross-reactive peptide. However, since CRF is a small peptide, the number of antigenic sites must be rather limited and the antiserum of the present experiment did not cross-react with various brain, pituitary and gut peptides. In addition, the CRF immunoreactive peptides in different ovine tissues have a molecular weight similar to the in vitro synthesized peptide (Fig. 3). It is likely, therefore, that the immunoreactivity measured in our study is due to CRF.

No CRF-I was detected in extrahypothalamic tissues including the CNS and gastro-enteropancreatic tract in the rabbit using antiserum to oCRF (Fischman & Moldow 1982). On the other hand, CRF-I has been found in both ovine and rat extrahypothalamic brain (Côté et al. 1982; Hashimoto et al. 1982). Thus, the discrepancy between the present data and those of Fischman & Moldow (1982) may be ascribed to species difference in CRF. A recent report has demonstrated that the primary structure of rat CRF has an 83% sequence homology with oCRF and antisera developed...
against whole oCRF sequence showed poor reactivity with the rat peptide (Rivier et al. 1983). We also examined the contents of CRF-I in rat organs by the same extraction method. The overall content was less than that found in ovine organs: hypothalamus 18.5 ± 2.0, cerebrum 12.5 ± 0.5, stomach 4.0 ± 1.0, duodenum 6.5 ± 1.5 and pancreas 3.5 ± 0.5 ng/g wet weight tissue, mean ± SEM (unpublished data).

The concentrations of CRF-I observed in this study are comparable with those reported by Côté et al. (1982) with the ovine brain. Although the CRF-I content of the hypothalamus in the present study is lower than that of the median eminence reported by Côté et al. (1982), this is due to the exclusion of median eminence from the tissue of the hypothalamus. The highest concentration of CRF-I in the pituitary might be ascribed to the accumulation of CRF released from the median eminence caused by stress during sampling. However, CRF-I was recently detected in the somatotrophs of the human pituitary by an immunoperoxidase technique (Kruseman et al. 1984).

In this study, only one peak of CRF-I was identified by gel chromatography of the cerebral and upper jejunal extracts. Linton & Lowry (1982) reported a small CRF-I in the void volume of gel filtration with Sephadex G-50 of ovine stalk median eminence extract. They suggested that this immunoreactivity was caused by a non-specific binding of CRF-41 to larger molecular weight proteins. Since there were large amounts of proteins in the void volume and their interference to RIA was also probable, we measured a non-specific binding for each fraction. The primary structure of ovine prepro-CRF was identified recently by the cloning of cDNA (Furutani et al. 1983). The precursor protein consists of 190 amino acids including two paired basic amino acid residues (Arg-Arg at positions 116 and 117 and Lys-Arg at positions 127 and 128) in addition to the Arg-Lys-Arg-Arg sequence at 144-147 immediately preceding CRF. It seems plausible to assume that the prohormone is proteolytically cleaved at one or both of these sites to produce novel peptides. Although Arg-Lys-Arg-Arg is a most accessible position for post translational processing, it is possible that the present antiserum did not react with any larger or smaller peptides.

The role of CRF in extra-hypothalamic tissues remains to be clarified. Other pituitary hormone releasing factors such as TRH and GRF have been found in extrahypothalamic tissues (Morley et al. 1977; Guillemin et al. 1982). Synthetic oCRF infused into the isolated perfused rat pancreas did not influence insulin and glucagon secretion, although the species difference in the biological activity of CRF can not be excluded (unpublished data).

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References


