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Abstract

The stability of rat (human) CRF in serum, urine and tissue incubation medium was examined using Sephadex gel filtration and CRF radioimmunoassay with anti-rat (human) CRF serum. Human serum after incubation with rat (human) CRF for 1 h at 37 degrees C showed two peaks of CRF immunoreactivity on a Sephadex G-50 fine column. Most of the immunoreactivity coeluted with the rat (human) CRF marker. When rat (human) CRF was incubated with rat liver, kidney or hypothalamus, only 3.1-14.9% of the CRF was recovered at the rat (human) CRF position on gel filtration, and two to four CRF-immunoreactive peaks appeared after the rat (human) CRF marker. When rat (human) CRF was incubated with human urine (pH 6.0) for 24 h at room temperature, one peak of CRF immunoreactivity coeluted with the rat (human) CRF marker on Sephadex gel filtration. The urine extracts of normal rats showed some small peaks of CRF-like immunoreactivity on the Sephadex column, with the main peak appearing after authentic CRF. These results suggest that rat (human) CRF is relatively stable in serum and urine, but is easily degraded by tissue enzymes, with the degraded CRF fragments being excreted in the urine.

KEYWORDS: rat(human) corticotropin releasing factor, sephadex chromatography, stability

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STABILITY OF RAT (HUMAN) CORTICOTROPIN-RELEASING FACTOR IN SERUM, URINE AND TISSUE INCUBATION MEDIUM

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Abstract. The stability of rat (human) CRF in serum, urine and tissue incubation medium was examined using Sephadex gel filtration and CRF radioimmunoassay with anti-rat (human) CRF serum. Human serum after incubation with rat (human) CRF for 1 h at 37 ℃ showed two peaks of CRF immunoreactivity on a Sephadex G-50 fine column. Most of the immunoreactivity coeluted with the rat (human) CRF marker. When rat (human) CRF was incubated with rat liver, kidney or hypothalamus, only 3.1-14.9 % of the CRF was recovered at the rat (human) CRF position on gel filtration, and two to four CRF-immunoreactive peaks appeared after the rat (human) CRF marker. When rat (human) CRF was incubated with human urine (pH 6.0) for 24 h at room temperature, one peak of CRF immunoreactivity coeluted with the rat (human) CRF marker on Sephadex gel filtration. The urine extracts of normal rats showed some small peaks of CRF-like immunoreactivity on the Sephadex column, with the main peak appearing after authentic CRF. These results suggest that rat (human) CRF is relatively stable in serum and urine, but is easily degraded by tissue enzymes, with the degraded CRF fragments being excreted in the urine.

Key words : rat (human) corticotropin releasing factor, sephadex chromatography, stability.

Vale *et al.* (1, 2) first clarified the structure of ovine CRF in 1981. In 1983, Shibahara *et al.* (3) clarified the structure of human prepro-CRF and reported that the structure of human CRF was different from ovine CRF by seven amino acids. Rivier *et al.* (4) found that the structure of rat CRF was identical to human CRF.

It has been reported that the half life of injected ovine CRF is relatively long in circulating human plasma (5-7). In the present study here we examined the stability of rat (human) CRF in human serum and urine and in the incubation medium of rat tissues, using Sephadex gel filtration and CRF radioimmunoassay with anti-rat (human) CRF serum developed in our laboratory.

MATERIALS AND METHODS

Incubation of rat (human) CRF. One hundred nanograms of rat (human) CRF was incubated in 1 ml of fresh human serum at 37 ℃ for 1 h. A sample of the serum (0.5 ml) was chromato-

graphed on a Sephadex G-50 fine column (0.9 × 60 cm). The same amount of serum was also chromatographed immediately after the addition of rat (human) CRF to serum without incubation. Serum without CRF was also chromatographed as a control.

Two hundred nanograms of rat (human) CRF was incubated with approximately 200 mg of rat hypothalamic tissue, liver and kidney slices in 2 ml of Dulbecco's modified Eagle Medium (DMEM) for 1 h at 37℃. A half milliliter of each incubation medium was applied to the Sephadex G-50 fine column.

Eight hundred nanograms of rat (human) CRF was incubated in 10 ml of urine (pH 6.0) or acidified urine (pH 1.5) at room temperature for 24 h. Urine samples (1 ml) were applied to the Sephadex G-50 fine column. In another experiment, 100 ng of rat (human) CRF was incubated in 10 ml urine (pH 6.0 or 1.5) and extracted with Vycore glass to examine the stability of rat (human) CRF during the extraction procedure. Vycore glass (250 mg) was added to urine and mixed. After centrifugation the Vycore glass was washed twice with 3 ml of water, and CRF was eluted with acetone-0.25 N HCl (1 : 1). The extract was lyophilized for chromatography. Urine from normal rats collected 4 h before and after injection of rat (human) CRF (10 μg/rat) was extracted by Vycore glass extraction and chromatographed.

Sephadex G-50 chromatography. Samples were applied to a Sephadex G-50 fine column (0.9 × 60 cm) and eluted in the CRF radioimmunoassay buffer (0.02 M PO_4 buffer, 0.15 M NaCl, 0.1 mM ascorbic acid and 0.5 % BSA, pH 7.4). Two milliliter fractions were collected, and the CRF in 0.3 ml of each fraction was radioimmunoassayed in duplicate.

Anti-rat CRF serum preparation. Two milligrams of synthetic rat (human) CRF purchased from Bachem Inc. (Torrance, CA) was conjugated to 6 mg of bovine serum albumin (Sigma) with 0.021 M glutaraldehyde. Japanese white rabbits were immunized by injecting 0.5 mg CRF-BSA conjugate in complete Freund's adjuvant every two weeks into multiple dorsal sites. After 15-17 weeks of treatment the animals were anesthetized and blood was collected from the carotid artery.

Radioimmunoassay of CRF. Iodination of rat (human) CRF was carried out using the same chloramine T method that we used for iodination of ovine CRF (8). The standard CRF or unknown sample (100 μl) was preincubated overnight at 4℃ with 200 μl of assay buffer and

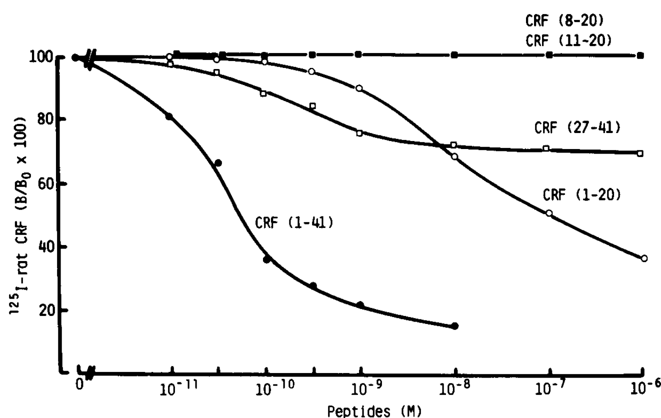

Fig. 1. Displacement of ^{125}I-rat (human) CRF from anti-rat (human) CRF serum by rat (human) CRF (1-41) and its fragments. Antiserum dilution, 1 : 7,500.

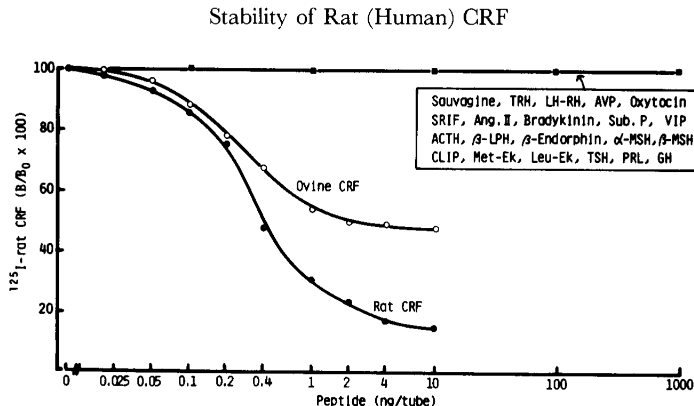

Fig. 2. Displacement of ^{125}I-rat (human) CRF from anti-rat (human) CRF serum by rat (human) and ovine CRF and other neuronal peptides. Antiserum dilution, 1 : 7,500.

100 μl of antiserum (final dilution 1 : 7,500). Tracer (ca 10,000-15,000 cpm) was added, and incubation was continued for 48 h. Separation of the free and antibody-bound CRF was performed after overnight incubation with 100 μl of normal rabbit serum (1 : 150) and 100 μl of anti-rabbit γ-globulin serum (1 : 30), followed by centrifugation for 20 min at 1,200 g (4 ℃).

The standard curve ranged from 20 pg/tube to 10 ng/tube. The intraassay coefficient of variation was 2.1 % for 463.2 pg, and the interassay coefficient of variation derived from seven independent assays using rat hypothalamic extract was 7.1 %.

Crossreactivities with human CRF (1-20), CRF (8-20), CRF (11-20) and CRF (27-41) were 0.3, 0, 0 and less than 0.003 %, respectively (Fig. 1). Crosseractivity with ovine CRF was 12.8 %, whereas there was no crossreactivity with sauvagine, TRH, LH-RH, somatostatin, arginine vasopressin (AVP), oxytocin, angiotensin II, bradykinin, vasoactive intestinal peptide, substance P, ACTH, β-lipotropin, β-endorphin, α-MSH, β-MSH, CLIP, met-enkephalin, leu-enkephalin, rat GH, TSH or prolactin (Fig. 2).

Synthetic rat (human) CRF and ovine CRF were purchased from Bachem Co, (Torrance, CA). Human CRF fragments were kindly supplied by Dr, N. Yanaihara (Shizuoka, Japan).

RESULTS

Stability of rat (human) CRF in serum. The half milliliter serum samples without CRF showed one small peak of CRF-like immunoreactivity near the void volume. The serum chromatographed immediately after adding rat (human) CRF showed two peaks of CRF immunoreactivity (Fig. 3). The main immunoreactivity (90.9 % of added CRF) coeluted with the rat (human) CRF marker, and another small peak eluted near the void volume. The serum incubated with rat (human) CRF for 1 h at 37 ℃ showed a similar elution pattern in CRF immunoreactivity to non-incubated serum on Sephadex gel filtration.

Stability of rat (human) CRF in incubation medium with rat tissues. When rat (human) CRF (200 ng) was incubated with rat hypothalamus in 2 ml of DMEM and 0.5 ml of the medium was chromatographed on a Sephadex G-50 fine column, a small peak appeared at the position of rat (human) CRF, but only 14.9 % of the CRF

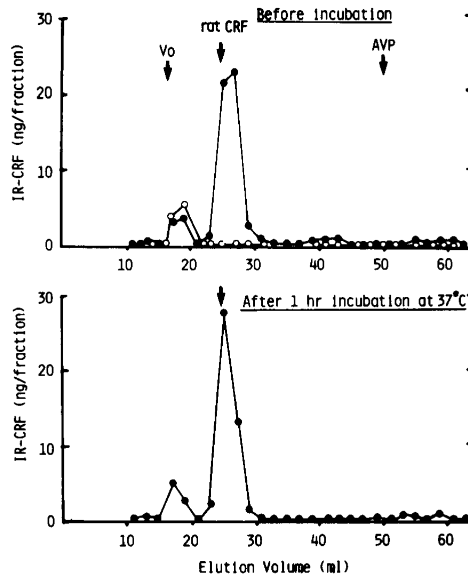

Fig. 3. Sephadex G-50 fine (0.9 × 60 cm) chromatography of rat (human) CRF before and 1 h after incubation in human serum at 37 ℃. One milliliter of serum (100 ng CRF equivalent) was applied to the column. ●——●, serum with CRF, ○——○, serum without CRF.

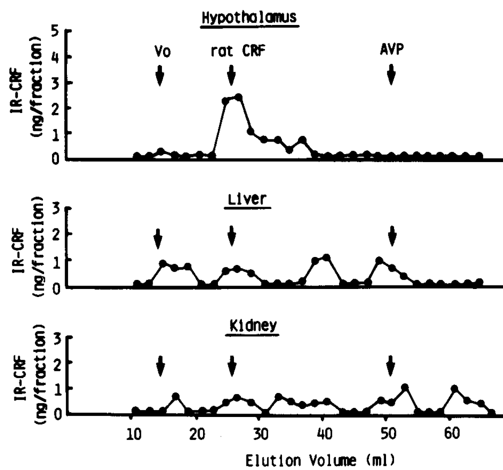

Fig. 4. Sephadex G-50 fine (0.9 × 60 cm) chromatography of rat (human) CRF in incubation medium with rat hypothalamus, liver and kidney. A half milliliter of medium (50 ng CRF equivalent) was applied to the column.

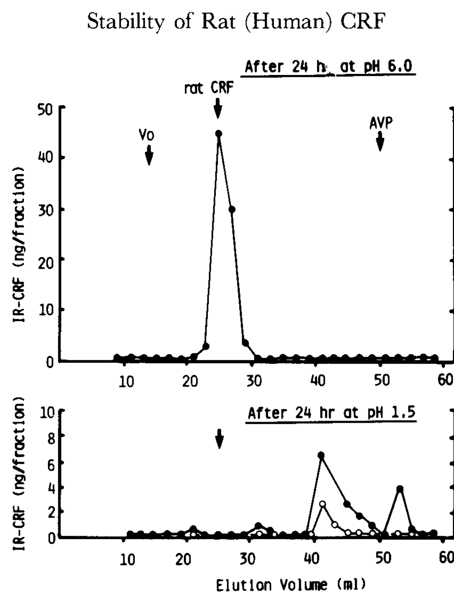

Fig. 5. Sephadex G-50 fine (0.9 × 60 cm) chromatography of rat (human) CRF 24 h after incubation in human urine (pH 6.0 and 1.5) at room temperature. One milliliter of urine (80 ng CRF equivalent) was applied to the column. ●——●, urine with CRF, ○——○, urine without CRF.

was recovered at the rat CRF position, and two small peaks appeared after rat (human) CRF (Fig. 4).

When rat (human) CRF was incubated with the rat liver, several peaks of CRF-like immunoreactivity appeared on gel filtration. The first one appeared near the void volume, and the second appeared at the position of the rat (human) CRF marker. The other three peaks appeared between the CRF and AVP markers. Only 3.5 % of the CRF was recovered at the rat (human) CRF positions.

When rat (human) CRF was incubated with rat kidney, most of the CRF was degraded, and several small peaks of CRF-like immunoreactivity appeared on gel filtration. The first two peaks appeared near the void volume and the rat (human) CRF marker, and the remaining peaks appeared at various other positions.

Stability of rat (human) CRF in urine. When human urine (pH 6.0) was incubated with rat (human) CRF for 24 h at room temperature and chromatographed on a Sephadex G-50 fine column, one peak of CRF immunoreactivity coeluted with the rat (human) CRF marker (Fig. 5). No other significant CRF-immunoreactive peak appeared. Almost all (97.9 %) of the rat CRF was recovered on chromatography. However, when the rat (human) CRF was incubated in acidified urine (pH 1.5), there was no CRF-like immunoreactive peak at the rat (human) CRF marker position, but two smaller molecular CRF-like immunoreactivities appeared. Forty-five percent of the CRF was recovered by chromatography.

When urine (pH 6.0 and 1.5) was extracted with Vycore glass and chromatographed on a Sephadex column, similar elution patterns of CRF-like immunoreactivity

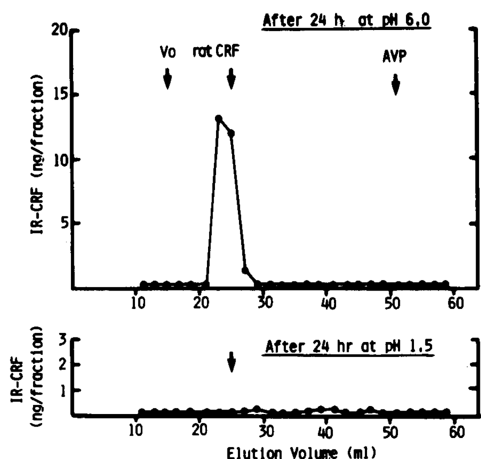

Fig. 6. Sephadex G-50 fine (0.9 × 60 cm) chromatography of rat (human) CRF 24 h after incubation with human urine (pH 6.0 and 1.5). Ten milliliters of urine (100 ng rat CRF equivalent) was extracted with 250 mg Vycore glass, and the extract was applied to the column.

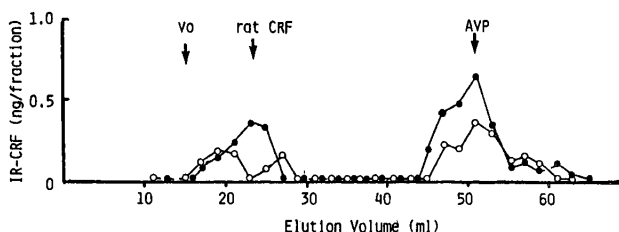

Fig. 7. Sephadex G-50 fine (0.9 × 60 cm) chromatography of the CRF-like immunoreactivity in rat urine collected 4 h before and after rat (human) CRF (10 μg/rat) injection. The urine was extracted with Vycore glass. ○——○, urine before CRF (10 μg) injection, ●——●, urine after CRF injection.

to non-extracted urine appeared, and 26.9 % of the added CRF was recovered at the rat (human) CRF marker position (Fig. 6).

When the urine of rats collected 4 h before and after injection of rat (human) CRF was extracted with Vycore glass and chromatographed, several CRF-immunoreactive peaks appeared. Small peaks appeared near the rat (human) CRF marker position, and other peaks appeared near the AVP marker position (Fig. 7). The smaller molecular CRF-like immunoreactive peak increased after rat (human) CRF injection.

DISCUSSION

We have developed an anti-rat (human) CRF serum to measure rat (human) CRF. The antiserum binds rat CRF more specifically than anti-ovine CRF, enabling us to measure rat (human) CRF more accurately.

The present results indicate that rat (human) CRF is relatively stable in serum and urine. Although additional separation techniques and CRF bioassay are needed to confirm this interpretation, the present results at least indicate that the molecular weight of rat (human) CRF did not change enough to be detected by Sephadex G-50 fine chromatography. The small loss of CRF immunoreactivity during incubation with serum and urine may account for the prolonged half life of injected CRF in circulating blood. Although rat (human) CRF was stable in natural urine, when it was incubated with acidified urine, no CRF-like immunoreactivity appeared at the position of the rat (human) CRF marker, and smaller molecular CRF-like immunoreactivities appeared. These results suggest that CRF was degraded at low pH.

The adsorption rate of ^{125}I-rat (human) CRF to Vycore glass in urine (pH 6.0) and its final extraction rate from urine were $32.9 \pm 0.4\%$ and $31.7 \pm 1.0\%$, respectively (unpublished data). Therefore, the present result (Fig. 6) showed that Vycore glass-extracted CRF was non-degraded CRF. The extracted rat urine showed a very small peak of CRF-like immunoreactivity, and the main CRF-like immunoreactivity eluted later than the authentic rat CRF marker. Although rat (human) CRF seemed to be stable in serum and urine, it was easily degraded by incubation with liver, kidney or hypothalamus. The results suggest that rat (human) CRF is degraded by tissue enzymes, and that the degraded CRF fragments are excreted in urine.

Human serum, urine and rat tissue incubation medium showed slight CRF-like immunoreactivity near the void volume. This immunoreactivity might be a precursor of CRF, aggregated CRF or a protein artifact. A combination of the latter two possibilities appears most likely.

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