PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **152**, 691-694 (1976)

# Corticotropin Releasing Factor (CRF) Activity in Rat Plasma[1] (39468)

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There have been several reports of corticotropin releasing activity in extrahypothalamic tissues and fluids (1-6). Indirect evidence of extrahypothalamic CRF was provided in cross-circulation experiments conducted in rats by Brodish and Long (2). Further indirect evidence of extrahypothalamic activity was also established by Egdahl (3) who found that dogs with pituitary islands continued to secrete ACTH despite removal of the entire nervous system.

Direct evidence for extrahypothalamic "tissue" CRF was first shown by Lymangrover and Brodish (7) who found that peripheral plasma taken from stressed, hypophysectomized rats with median eminence lesions had greater CRF activity than plasma from similar nonstressed animals. In these experiments CRF activity of the donor rat plasma was assayed in median eminence-lesioned animals which had been shown to respond to median eminence extract but not to cerebral cortical extract or to ether stress. The tissue CRF and hypothalamic CRF were similar in that both stimulated corticoid secretion in assay rats. However, tissue CRF and hypothalamic CRF differed in the time courses of effect in the assay rats. Hypothalamic CRF activity provoked a transient early effect whereas the effect of tissue CRF was found to be prolonged.

Plasma has also been reported to stimulate ACTH release from rat pituitary cells maintained in monolayer culture (6). As little as 0.1% v/v plasma in the assay system stimulates ACTH release.

During the course of studies of hypothalamic CRF activity we observed a similar effect of peripheral plasma on incubated, dispersed pituitary cells prepared by the method of Swallow and Sayers (8). The present report describes efforts to further characterize plasma CRF activity.

*Preparation of isolated pituitary cells.* Four to six male Sprague-Dawley rats, weighing 200-300 g were used in each experiment. After decapitation the anterior pituitaries were removed, rinsed in normal saline, blotted, quartered, and placed in a 50-ml Erlenmeyer flask containing 20 ml of Krebs-Ringer bicarbonate buffer with 0.2% glucose (KRBG) and 0.5% trypsin (Worthington Biochemical Corp., Freehold, N.J.) (8). The tissue pieces were agitated at 37° in a 95% $O_2$:5% $CO_2$ atmosphere. After 20 min the supernatant containing dispersed cells was transferred to an iced flask, fresh KRBG with trypsin was added to the remaining fragments and the above process was repeated. After three washes, the combined supernatants were centrifuged at 100*g* for 30 min at 4°. The pellet containing the cells was resuspended in KRBG containing 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and 30 mg of lima bean trypsin inhibitor (Worthington Biochemical Corporation, Freehold, N.J.), yielding a final concentration of 0.5 pituitary equivalents per milliliter. Cell suspension aliquots of 0.5 ml containing approximately $10^5$ cells were preincubated in 10-ml Teflon beakers for 10 min at 37° until the test substances were added.

*Preparation and additions of test substances.* The following test substances were added: lyophilized rat hypothalamic extract (RHE) (NIAMD-rat HE-RP-1), rat plasma or serum, heparin, and rat plasma fractionated as described below. All test substances were adjusted to 500-$\mu$l volumes with KRBG and added to the cell suspension, yielding a final incubation volume of 1 ml. RHE was dissolved in 2 ml of 0.2 *N* acetic acid, aliquoted into 100-$\mu$l vials, and frozen until use. The aliquots were thawed and diluted with buffer before addition to the test system. Blood was collected from rats

[1] Supported in part by Institutional Research Support from the Veterans Administration and by NIAMDD Grant AM01457.

[2] MRIS No. 4883.



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by rapid decapitation (unstressed) or by exsanguination after 5 min exposure to ether (stressed). Samples were collected on ice, centrifuged at 100*g*, and frozen until use. The possible CRF activity of heparin (sodium heparin, Invernex, 1000 USP units per milliliter) was tested by introduction into the cell incubation at the same concentration as that used to heparinize blood (10 units/ml).

*Fractionation of plasma.* Two methods were employed (i) 2 ml of plasma was introduced onto a 1.5 × 30-cm G-75 Sephadex column and eluted with an 0.05 *M* phosphate buffer (pH 7.4) at 25°. (ii) 10 ml of plasma separated by vacuum ultrafiltration at 50 Torr at 4° using a membrane with an average molecular weight retention of 15,000. Both the retained fraction (retenate) and the ultrafiltrate were collected and stored at 4° or until assayed. In both experiments a portion of the raw plasma was assayed to determine recovery.

*Assay of ACTH.* ACTH released into the incubation medium was determined by radioimmunoassay as previously described (9). The antibody employed is highly specific for $\alpha_h$-ACTH and $\alpha^{11-24}$ ACTH; reacting with both on an equimolar basis. It fails to cross-react significantly with the following polypeptides: $\alpha$-MSH, $\beta_p$-MSH, $\beta_h$-MSH, $\alpha_p{}^{17-39}$ACTH, $\alpha_h{}^{17-39}$ACTH, and $\alpha_p{}^{25-39}$ ACTH and $\alpha_h{}^{25-39}$ ACTH or $\alpha^{1-16NH_2}$ ACTH. The sensitivity limit of the assay is 30 pg/ml. In the present studies the measured quantities of ACTH were in the nanogram per milliliter range.

*Results.* Dose-response relationships in the isolated pituitary cell system were established using the standard NIAMDD rat hypothalamic extract as a stimulus. The results of this experiment are shown in Fig. 1. The lower asymptote of the dose-response curve was used to estimate the sensitivity of the assay system, which was about 0.01 mg RHE. Maximal stimulation was achieved by 1.0 mg RHE which caused release of 10 ng ACTH/$10^5$ cells during the 30 min incubation period. The ACTH-releasing activity of 0.1 mg RHE was chosen as a standard for the studies.

The time course of ACTH release in response to 0.1 mg RHE is depicted in Fig. 2.

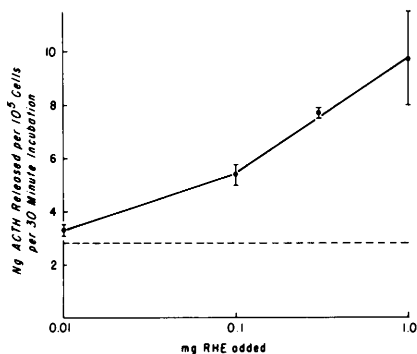


FIG. 1. Dose-response effect of rat hypothalamic extract (RHE) on ACTH release from dispersed pituitary cells.

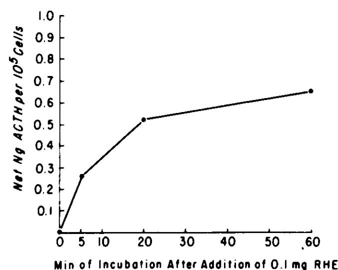


FIG. 2. Time-course of rat hypothalamic extract (RHE)-induced ACTH release by dispersed pituitary cells.

Approximately one-half of the total net ACTH release occurred within 5 min after addition of the RHE as previously observed by Takebe *et al.* using the monolayer culture technique (6). After 30 min, 85% of the total net ACTH release had occurred and this duration of incubation was routinely employed. It was determined that a 5-min period of stabilization of the system before the addition of the test substance (preincubation period) was technically convenient.

The stimulatory effects of graded volumes of rat plasma and serum are shown in Table I. Both serum and plasma produced dose-related ACTH release. Although rat plasma had approximately twice the ACTH releasing potency of serum at the highest tested dose the differences were not statistically significant. Since the plasma was prepared with heparin and the serum was not, the possible ACTH-releasing effect of heparin was assessed by testing heparin alone and by heparinizing serum. In two experiments

TABLE I. EFFECT OF RAT PLASMA AND SERUM ON IN VITRO PITUITARY ACTH RELEASE.

| Test substance | Amount added ($\mu$l) | Number of replications | Net ACTH release[a] (ng/$10^5$ cells/30 min) |
|---|---|---|---|
| Plasma | 100 | 8 | 4.7 ± 1.4 |
| | 10 | 6 | 1.1 ± 0.6 |
| | 1 | 4 | 0.5 ± 0.4 |
| Serum | 100 | 3 | 1.9 ± 1.8 |
| | 10 | 2 | 1.5, 0 |
| | 1 | 2 | 0, 0 |
| Plasma (unstressed) | 100 | 4 | 5.3 ± 2.3 |
| Plasma (stressed) | 100 | 4 | 4.7 ± 1.7 |

[a] Mean ± SEM.

heparin alone had no ACTH-releasing activity (data not shown).

In four experiments the effects of plasma obtained from stressed and unstressed rats were compared. As indicated in Table I both produced ACTH release, but the difference between the two was not significant.

The ACTH-releasing activity of plasma was further characterized by ultrafiltration and gel separation. After ultrafiltration using a membrane with an average molecular weight retention of 15,000, 93% of the ACTH releasing activity was found in the retentate as shown in Table II. Plasma was also separated by using G-75 Sephadex and the collected fractions tested for ACTH-releasing activity (Fig. 3). The fractions with ACTH-releasing activity were not retarded by the Sephadex. The findings of these studies suggest that the CRF activity of plasma is associated with a molecular weight greater than 15,000.

*Discussion.* These studies confirm the presence of an ACTH-releasing substance in rat plasma and suggest it is, or is associated with, a large molecule. Both stressed and unstressed rat plasma and serum caused ACTH release from dispersed rat pituitary cells, supporting the observation of Takebe *et al.* (6) who used a pituitary monolayer cell culture system. The present studies extend their observations by showing with two different separation methods, that the substance in plasma behaves like a molecule of a molecular weight of greater than 15,000.

The CRF activity of rat plasma (and serum) is like hypothalamic CRF in that the pituitary response is dose-related. However, it is apparently not identical to hypothalamic CRF because the dose-response curves of the two materials were found to be different in Takebe's studies (6) and because the CRF activity in plasma is not altered by factors ordinarily expected to affect pituitary-adrenal function, such as stress. Finally, it can be calculated that the plasma compartment contains many more times the CRF activity than that of the hypothalamus. In the present studies as little as 100 $\mu$l of rat plasma had approximately the same ACTH releasing activity as the equivalent extract of 0.5 rat hypothalamus. The above data would suggest a nonhypothalamic source of plasma CRF.

It is also possible that plasma CRF is a purely artifactual phenomenon. Both the dispersed pituitary cell technique of Sayers and the monolayer culture technique employ cells that are exposed directly to the stimulatory agent. Under these circumstances the cells do not have the protection normally afforded by the selective barrier of

TABLE II. THE EFFECT OF RAT PLASMA FRACTIONATED BY ULTRAFILTRATION ON *in Vitro* PITUITARY ACTH RELEASE.

| Fraction | Volume added ($\mu$l) | Net ACTH released[a] (ng/$10^5$ cells/30 min) Experiment 1 | Experiment 2 |
|---|---|---|---|
| Dialysate | 100 | 1.1 | 1.2 |
| | 10 | 0 | 1.0 |
| | 1 | 0 | 0 |
| Retentate | 100 | 13.2 | 9.7 |
| | 10 | 5.7 | 3.2 |
| | 1 | 1.0 | 1.2 |

[a] Results of two experiments.

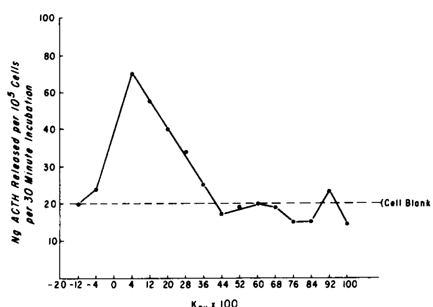


FIG. 3. Effect of G-75 Sephadex-fractionated rat plasma on dispersed pituitary cells.

the capillary wall and associated structures between plasma and the secretory cell and the plasma. Thus, plasma may exert a nonphysiological effect directly on the cells. This explanation would account for the apparent discrepancy between the relative potencies of hypothalamic CRF and plasma CRF. Furthermore, the explanation might account for the apparently previously inexplicable phenomenon of Egdahl (3) in which dogs with isolated pituitaries following complete brain removal were found to elaborate massive amounts of ACTH. It is possible that the brainectomized dog has increased pituitary capillary permeability and plasma factors not ordinarily reaching the pituitary cells, such as plasma, may come into direct contact with cells and cause ACTH release. The biochemical nature of plasma CRF and its physiological and/or pathophysiological role awaits further study.

*Summary*. The ACTH-releasing activity of hypothalamic extract and rat plasma was examined with the dispersed rat pituitary cell technique of Swallow and Sayers (8). Although both plasma and serum caused ACTH release which was dose-related, stress did not enhance the ACTH releasing activity. Furthermore, separation studies of plasma using ultrafiltration and gel separation suggest that the CRF activity in plasma is associated with molecules of a molecular weight greater than 15,000.

The hypothalamic extract used in this study was kindly provided by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD) Rat Pituitary Hormone Distribution Program.

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Received January 30, 1976 P.S.E.B.M. 1976, Vol. 152.