

Ultrashort-loop positive feedback of corticotropin (ACTH)-releasing factor to enhance ACTH release in stress

(ovine corticotropin-releasing factor/plasma ACTH/third ventricle/hypothalamus)

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ABSTRACT Previous experiments have shown that intraventricular injection of ovine corticotropin (ACTH)-releasing factor (oCRF) in doses too low to elevate plasma ACTH by direct action on the pituitary does not lower plasma ACTH, suggesting that the peptide lacks a negative ultrashort-loop feedback action to suppress its own release under resting conditions. The present study was performed to determine whether oCRF has any action to alter CRF release in stress. The peptide was injected into the third ventricle or external jugular vein of freely moving ovariectomized female rats 5 min prior to application of ether stress. When oCRF was injected into the third ventricle in doses of 500 pg (0.1 pmol) or less, there was no significant alteration in plasma ACTH prior to ether stress; however, there was a significantly enhanced increase in plasma ACTH 2 and 5 min after ether stress applied 5 min after intraventricular injection of oCRF at doses of 50 (0.01 pmol) or 150 pg (0.03 pmol). These results suggest that the peptide acts on structures adjacent to the third ventricle to augment stress-induced CRF release. To rule out the possibility that the sensitivity of the pituitary itself to CRF increases dramatically following stress, 10 or 100 ng of oCRF was injected i.v. These doses produced a significant dose-related increase in plasma ACTH at 2, 5, or 15 min. In other groups receiving the same doses of oCRF and ether stressed 5 min later, plasma ACTH was significantly higher 2 or 5 min after ether stress when compared with plasma ACTH in ether-stressed saline-injected animals. However, in contrast with the results of intraventricular injection of oCRF, the release of ACTH was no greater than that obtained by summing the independent effects of exogenous oCRF and the CRF released by stress. We conclude that CRF may have a positive ultrashort-loop feedback action to enhance stress-induced ACTH release and that this enhancement is not due to increased sensitivity of anterior pituitary corticotrophs to CRF.

Ultrashort-loop feedback of releasing hormones to alter their own release was postulated a number of years ago by Martini and co-workers (1). There now appears to be a number of examples of this type of interaction of peptides within the brain to alter their own release. The existence of an ultrashort-loop feedback mechanism by which somatostatin alters its own release has been suggested (2). Similarly, intraventricular injection of oxytocin appears to inhibit its own release (3).

Consequently, we have looked for possible interactions of corticotropin (ACTH)-releasing factor (CRF) with other peptides in the hypothalamus. We found to our surprise that injection of ovine CRF (oCRF) into the third ventricle decreases plasma growth hormone with a minimal effective dose of 0.1 nmol. A higher dose (1.0 nmol) also decreased plasma luteinizing hormone (LH) (4, 5). In both instances,

similar results have been obtained following lateral ventricular injection of the peptide (6, 7). Thus, it appears that the CRF neurons interact with those controlling the release of growth hormone and LH to suppress release of these hormones. By contrast, there was no effect of oCRF on the release of follicle-stimulating hormone or thyrotropin (4, 5).

In these experiments, intraventricular injection of oCRF produced an elevation in plasma ACTH levels in doses as low as 0.1 nmol of peptide. This elevation was presumably brought about by delivery of oCRF to the anterior pituitary and stimulation of ACTH release. The lowest dose (0.1 pmol) did not elevate ACTH release nor did it lower the already low plasma ACTH levels in these resting animals. Thus, no evidence for negative ultrashort-loop feedback of CRF to inhibit its own release was obtained (4, 5).

Therefore, we determined the effects of intraventricular injection of oCRF on plasma ACTH in stressed rats to look for the possible existence of ultrashort-loop feedback of CRF to alter ACTH release in stressful conditions. To our surprise, instead of finding evidence for a negative ultrashort-loop feedback of CRF to suppress its own release, we found evidence for a positive ultrashort-loop feedback of the peptide in animals stressed by brief anesthesia with ether.

MATERIALS AND METHODS

Adult female Sprague–Dawley (Holtzman, Madison, WI) rats weighing 180–200 g were used. They were housed in group cages under conditions of controlled temperature (23–25°C) and lighting (on from 0500 to 1900 hr) and provided rat chow and water ad lib. They were ovariectomized while anesthetized with ether to eliminate negative feedback of gonadal steroids and provide elevated levels of gonadotropins. This was so that these experiments would be comparable with our previous study in which we evaluated the effect of CRF on gonadotropin secretion. The animals were used for experiments 4–6 weeks after ovariectomy.

A silastic cannula was inserted into the right external jugular vein 24 hr before experimentation using the technique of Harms and Ojeda (8). To evaluate the effect of intravenous CRF, synthetic oCRF (Peninsula Laboratories, San Carlos, CA) dissolved in 0.3 ml of 0.9% NaCl (saline), or an equal volume of saline alone, was injected through the cannula. In non-stress experiments, sequential blood samples (0.9 ml) were withdrawn 0, 2, 5, 15, and 30 min after injection of oCRF in conscious freely moving rats. In experiments involving ether stress, oCRF or saline alone was injected through the jugular cannula 5 min prior to the application of 1 min of ether

Abbreviations: ACTH, corticotropin; CRF, corticotropin-releasing factor; oCRF, ovine CRF.

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stress in a counterbalanced design. Animals were ether stressed by placing them in a jar containing a layer of ether-saturated cotton on the bottom. Sequential blood samples were taken just before oCRF or saline injection and 2, 5, and 15 min after initiation of ether stress.

In rats to be injected into the third ventricle, a 23-gauge stainless steel cannula was implanted in the third ventricle 7–10 days before experimentation as described (5). The animals were then placed in individual cages. Synthetic oCRF in 2 μ l of saline or saline alone was microinjected into the third ventricle of conscious freely moving rats. Five minutes later, animals were ether stressed and heparinized blood samples were withdrawn as described above. Plasma was separated by centrifugation at low speed at 4°C and stored frozen until the day of assay.

Radioimmunoassay. Synthetic human ACTH and antibodies directed against it were provided by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. ACTH was labeled according to the method of Reese *et al.* (9). Plasma was extracted according to the method of Vaudry *et al.* (10). ACTH in 500 μ l of plasma was adsorbed to 40 mg of silicic acid and then eluted with 2 ml of acetic acid/acetone/water (1:40:59). The ACTH assay was carried out after evaporation of the sample under N₂ gas. The sensitivity of the assay was 10 pg per tube. Recovery of ACTH in plasma was 79.9% with an intraassay coefficient of variation of 10.6%.

Statistical Analysis. Statistical analysis was conducted by analysis of variance with repeated measure followed by the Student–Newman–Keuls multiple comparison test or by Student's *t* test after determining the area under the curve of plasma ACTH concentrations by planimetry. Results presented in the figures represent mean \pm SEM.

RESULTS

Non-Stress Experiments. It has been shown that injection of 500 pg of oCRF into the third ventricle has no effect on plasma ACTH level (4, 5). A lower dose (150 pg) of oCRF or saline alone injected into the third ventricle failed to alter the level of plasma ACTH in the present experiments (Fig. 1).

Intravenous injection of oCRF induced significant increases in plasma ACTH levels in a dose-related manner (Fig. 1). The maximal level of plasma ACTH was seen 2 min after

injection of oCRF at both doses. Plasma ACTH remained elevated at approximately the same level between 2 and 30 min after injection of the lower (10 ng) dose. By contrast the higher (100 ng) dose induced a higher peak level of plasma ACTH 2 min after injection and the level of plasma ACTH decreased gradually thereafter.

Stress Experiments. Administration of ether stress for 1 min was followed by an elevation in plasma ACTH levels in animals injected intraventricularly 5 min previously with saline (data not shown). When 50 ng of oCRF was injected into the third ventricle there was a significant elevation in plasma ACTH 5 min later ($P < 0.025$) and this value was also significantly higher than that of saline-injected animals ($P < 0.025$). Two minutes after ether stress, plasma ACTH levels were significantly higher ($P < 0.025$) in animals previously injected with 50 ng of oCRF than in animals injected with saline. This higher level after stress can be attributed to the higher initial levels of ACTH prior to ether stress because the injection of this dose of oCRF elevated plasma ACTH *per se*. The area under the curve of plasma ACTH level after ether treatment was similar in rats injected intraventricularly with this dose of oCRF and in control rats.

Since intraventricular injection of oCRF at a dose that by itself elevated the plasma ACTH level failed to alter the response to ether, in later experiments we decreased the dose of oCRF injected into the third ventricle to less than the threshold to elevate plasma ACTH. When doses of 50 pg (0.01 pmol) or 150 pg (0.03 pmol) of oCRF were injected there was no elevation in plasma ACTH 5 min later, just prior to administration of ether stress (Fig. 2). In these two groups, ether stress evoked a significantly greater elevation in plasma ACTH than the significant elevation observed in saline-injected controls at both 2 and 5 min after onset of ether stress. When the area under the curve of plasma ACTH level was determined in both groups by planimetry (Fig. 3), the increase was highly significantly greater in both CRF-injected groups as compared with controls.

To test the possibility that stress might in some way enhance the sensitivity of the corticotrophs to CRF and that this could be the mechanism by which intraventricular CRF produces further elevation of plasma ACTH levels in stressed rats, ether stress was administered to animals injected *i.v.* with oCRF, which would deliver the peptide to the pituitary directly. Doses of either 10 or 100 ng of oCRF injected *i.v.*

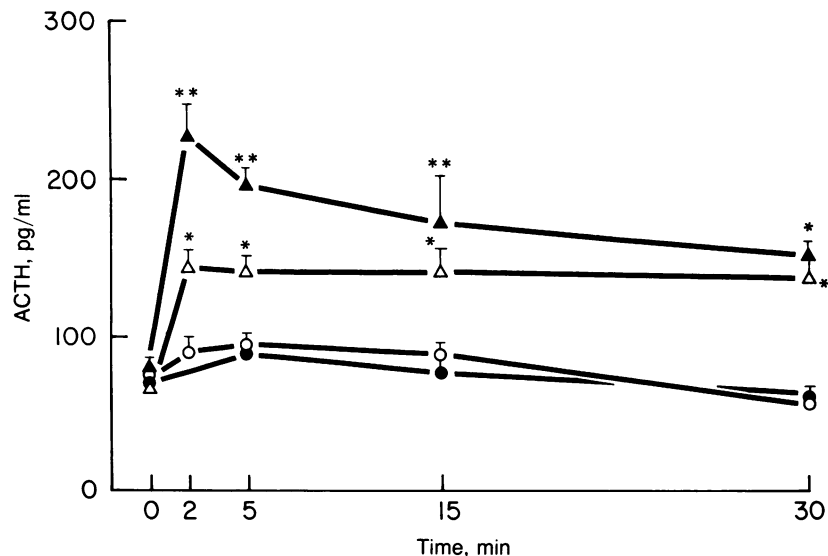


FIG. 1. Effect of injection of synthetic oCRF into the third ventricle or the jugular vein on plasma ACTH. ▲, 100 ng of CRF *i.v.* ($n = 5$); △, 10 ng of CRF *i.v.* ($n = 5$); ○, saline *i.v.* ($n = 6$); ●, 150 pg of CRF intraventricularly ($n = 6$). *, $P < 0.01$ vs. saline-injected controls; **, $P < 0.001$ vs. saline-injected controls.

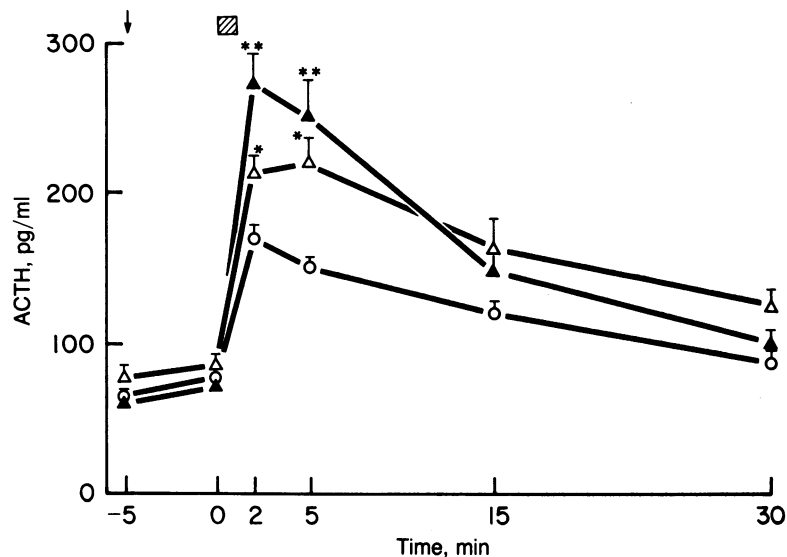


FIG. 2. ACTH release induced by ether stress (⊞) after injection of synthetic oCRF or saline into the third ventricle (↓). ▲, 150 pg of CRF ($n = 12$); △, 50 pg of CRF ($n = 11$); ○, saline ($n = 18$). *, $P < 0.05$ vs. saline-injected controls; **, $P < 0.01$ vs. saline-injected controls.

elicited significant increases in plasma ACTH 5 min after injection ($P < 0.05$, $P < 0.005$, respectively) (Fig. 4). Administration of ether stress elevated plasma ACTH levels in saline-injected controls. The elevation in the oCRF-injected animals was greater than that in the ether-stressed controls at 2 and 5 min. However, this increase was only additive above the already elevated levels in the saline-injected control animals and there was no evidence for a synergistic effect (Fig. 5). These results are in contrast to the significant further elevation in plasma ACTH levels after ether stress seen following third ventricle injection of lower doses of oCRF, which, by themselves, failed to alter plasma ACTH.

Behavioral Effects of oCRF Injected Intraventricularly. No difference in the time of awakening, as evidenced by righting and movement around the cage, was observed following ether anesthesia after i.v. injection of oCRF; however, it was noted that all animals injected intraventricularly with the higher (150 pg) dose of oCRF awakened within 1 min of

removal from the ether-containing jar. Control animals injected with saline intraventricularly did not awaken within 1 min. This decrease in the duration of ether anesthesia was not seen following the lower (50 pg) dose of intraventricular oCRF.

DISCUSSION

These results show that intraventricular injection of low doses of oCRF, which had no effect on plasma ACTH levels by themselves, enhanced the ACTH release induced by ether stress. The most likely explanation for these results is that there is an ultrashort-loop positive feedback mechanism whereby CRF neurons augment the release of CRF following stress. There could be recurrent collaterals of CRF neurons with perikarya in the paraventricular nucleus (11) that act back on these perikarya to augment CRF release under stressful but not resting conditions. Such a mechanism would enhance stress-induced CRF, and in turn ACTH release, and magnify the stress response. Such an action would be analogous to the positive feedback action of gonadal steroids

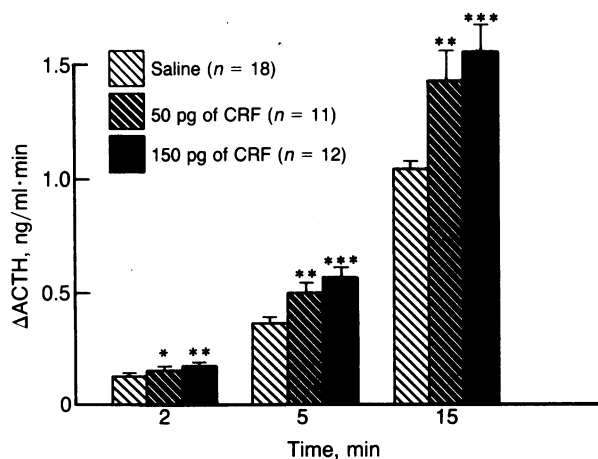


FIG. 3. Area under the plasma ACTH level curve (Δ ACTH) at various times after ether stress. To obtain the area, a horizontal line was drawn through the mean value of plasma ACTH at zero time (just prior to ether treatment) and the area above this line between zero time and the time chosen was determined by planimetry. *, $P < 0.025$ vs. saline-injected controls; **, $P < 0.005$ vs. saline-injected controls; ***, $P < 0.001$ vs. saline-injected controls.

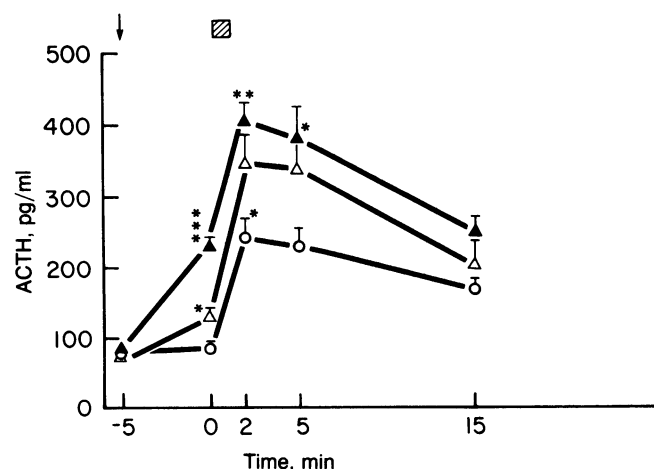


FIG. 4. ACTH release induced by ether stress (⊞) after i.v. injection of synthetic oCRF or saline (↓). ▲, 100 ng of CRF ($n = 5$); △, 10 ng of CRF ($n = 5$); ○, saline ($n = 6$). *, $P < 0.05$ vs. saline-injected controls; **, $P < 0.01$ vs. saline-injected controls; ***, $P < 0.005$ vs. saline-injected controls.

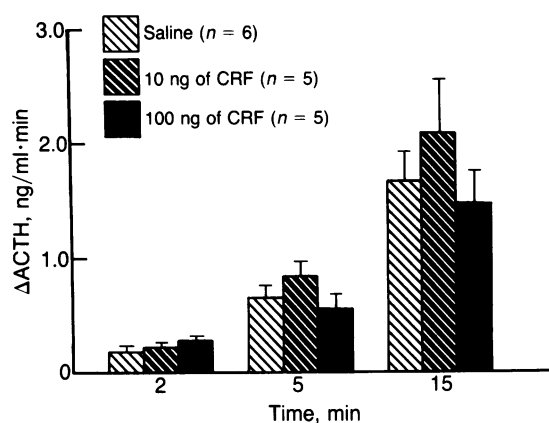


FIG. 5. Area under the plasma ACTH level curve (Δ ACTH) at various times after ether stress. To obtain the area, a horizontal line was drawn through the mean value of plasma ACTH at zero time (just prior to ether treatment), and the area above this line between zero time and the time chosen was determined by planimetry.

to enhance gonadotropin release and thereby bring about the preovulatory release of gonadotropins. There is electrophysiological evidence for recurrent collaterals of peptidergic neurons in the hypothalamus (12) and this has been demonstrated by electron microscopic immunocytochemistry in the case of luteinizing hormone-releasing hormone neurons (13).

It was considered possible that the apparent positive ultrashort-loop feedback of CRF might be accounted for by an enhanced sensitivity of the corticotrophs of stressed animals to CRF. If that were the case, small amounts of intraventricularly injected CRF reaching the pituitary after uptake by portal vessels, which were insufficient to stimulate the corticotrophs under resting conditions, might stimulate them under stress and give the enhanced ACTH release observed. Consequently, we performed experiments with i.v. injection of oCRF in doses that could stimulate the corticotrophs and then ether stressed the animals. Although the ACTH released under these conditions was greater than that released in animals previously injected with saline, there was no enhancement of ACTH release but only a summation of that produced by exogenous CRF and the stress-induced ACTH release. Therefore, it appears that there is no alteration in sensitivity of the corticotroph to CRF in the ether-stressed rat.

These results do not determine the precise site of action of oCRF to evoke additional CRF release during stress. It is likely that the action is on tissue adjacent to the third ventricle and it is noteworthy that this would bring the exogenous CRF in contact with the CRF neuronal system, which extends primarily from the paraventricular nucleus to the external layer of the median eminence (11). These are areas adjacent to the third ventricle.

It is possible that the action is not a direct one on the CRF neurons but may involve interneurons. It is even possible that the action may be induced indirectly since it has recently been shown that injection of CRF into the lateral ventricle can produce a discharge of epinephrine from the adrenal medulla (14). It is conceivable that this occurred in our experiments and that the epinephrine then acted directly on the pituitary to induce ACTH release (15) as postulated by Long (16). Recent studies indicating the presence of β_2 -adrenergic receptors in the anterior pituitary (17) and the ability of β -adrenergic agonists to augment ACTH release even in animals with hypothalamic lesions suggest that this may be a real possibility (18).

Since the animals injected with the higher dose of oCRF

into the ventricle awakened earlier than animals injected with the lower dose or with saline, it is also conceivable that the augmented ACTH release following ether stress in the CRF-injected animals might in some way be related to an alerting action of CRF directly on central nervous system neurons involved in inducing stress-induced CRF release. Indeed, Britton *et al.* (19) have reported that lateral ventricular injection of CRF produces behavioral activation. Although we think this unlikely in view of the fact that the augmentation was seen even with the lower dose of oCRF, which did not change the duration of ether anesthesia, it will be necessary to repeat these experiments using stresses in which the animals are unanesthetized to rule out this possibility completely. Furthermore, to localize the site of action of oCRF in augmenting its own release it will be necessary to microinject it into loci adjacent to the ventricle suspected of being the site of action. A likely location would be the paraventricular nucleus itself.

The ultrashort-loop positive feedback was not observed with intraventricular injection of a high (50 ng) dose of oCRF, which elevated plasma ACTH prior to application of ether stress. The failure to see the action under these conditions was probably related to the very high plasma ACTH level, which could not be elevated further by increased release of endogenous CRF. The plasma ACTH in these animals was as high as that seen following a maximal dose of oCRF in previous experiments.

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