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PROPERTIES AND REGULATION OF HIGH-AFFINITY PITUITARY RECEPTORS FOR CORTICOTROPIN-RELEASING FACTOR

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Specific receptors for corticotropin-releasing factor (CRF) were identified in the rat anterior pituitary gland by binding studies with ^{125}I-Tyr-CRF. Binding of the labeled CRF analog to pituitary particles was rapid and temperature-dependent, and reached steady state within 45 min at 22°C. The CRF binding sites were saturable and of high affinity, with dissociation constant (Kd) of 0.76 x 10^{-9} M. Pituitary binding of ^{125}I-Tyr-CRF was inhibited by CRF, Tyr-CRF and the active 15-41 fragment of CRF, but not by the inactive 21-41 CRF fragment and unrelated peptides. The binding-inhibition potencies of the CRF peptides were similar to their activities as stimuli of adrenocorticotropic hormone (ACTH) release. The high-affinity CRF sites were markedly reduced in adrenalectomized rats, and this change was reversed by dexamethasone treatment. These data indicate that the high-affinity CRF sites demonstrated in the anterior pituitary are the functional receptors which mediate the stimulatory action of the peptide on ACTH release, and that CRF receptors are down-regulated during increased secretion of the hypothalamic hormone.

The recently characterized hypothalamic corticotropin releasing factor (CRF) is a potent stimulus of ACTH release *in vivo* (1) and *in vitro* (2). Stimulation of ACTH release in isolated pituitary cells by nanomolar concentrations of CRF is accompanied by parallel increases in adenylate cyclase and cyclic AMP-dependent protein kinase, implying that specific receptors for the hypothalamic peptide are present in the plasma membrane of the corticotrophs (3). We have employed a radioiodinated CRF analog to identify and characterize specific binding sites for CRF in the rat anterior pituit-

Abbreviations: CRF, corticotropin releasing factor; ACTH, adrenocorticotrophic hormone, corticotropin; PBS, Dulbecco's phosphate-buffered saline; EGTA, ethylene glycol bis (β-aminoethyl ether)N,N,N',N'-tetraacetic acid; GnRH, gonadotropin releasing hormone.

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ary gland. These sites were found to exhibit the properties of physiologically relevant receptors which serve to mediate the regulatory action of CRF on ACTH release.

METHODS

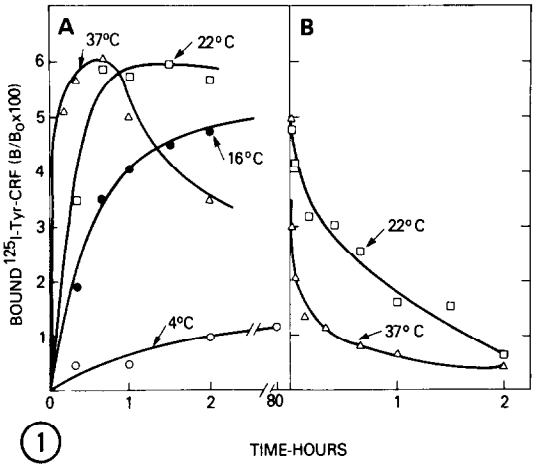
Ovine CRF, Tyr-CRF and the CRF fragments 15-41 and 21-41 were synthesized by solid-phase methodology on β-benzyhydrylamine, polystyrene 1% 1% divinyl benzene copolymer resin prepared by a modification of the method of Matsueda and Stewart (4). Other peptides were obtained from Peninsula Laboratories (San Carlos, CA) with the exception of angiotensin II, which was obtained from Beckman Bioproducts (Palo Alto, CA). ^{125}I-Tyr-CRF was prepared by lactoperoxidase iodination employing 5 µg of Tyr-CRF and 2.0 mCi of Na^{125}I. After 3 min, the reaction was stopped by addition of 200 µl of PBS containing 0.1% BSA and 0.1% sodium azide. The labeled CRF was then purified by gel filtration on a 1.5 x 90 cm Biogel P-10 column equilibrated with 50 mM sodium acetate buffer pH 5.5 containing 0.1% BSA. The specific activity of the labeled peptide ranged from 240 to 280 µCi/µg.

For receptor binding studies, 200 g male Sprague-Dawley rats (Charles River, Wilmington, MA) were killed by decapitation, and the anterior pituitary glands were dispersed in 50 mM Tris-HCl buffer pH 7.4 containing 5 mM $MgCl_2$ and 2 mM EGTA, by 6 strokes of a mechanical homogenizer (Teckmar Co., Cincinnati, OH). Homogenates were filtered through nylon gauze and centrifuged at 30,000 x g for 30 min, and the pellet resuspended in the same buffer to give a protein concentration of 200 to 250 µg in 100 µl. For the binding assay, 100 µl aliquots of the membrane suspension were incubated with 100,000 cpm of ^{125}I-Tyr-CRF (0.1 nM) in a total volume of 300 µl of 50 mM Tris-HCl buffer containing 5 mM $MgCl_2$, 2 mM EGTA, 0.1% BSA and 100 KIU/ml of aprotinin (Sigma Co., St. Louis, MO). Unless otherwise specified, incubations were performed for 60 min at 22°C. After incubation, the receptor-bound radioactivity was isolated by filtration through glass fiber filters (Whatman, Clifton, NJ) presoaked in 1% BSA. The filters were washed 3 times with 4 ml of cold PBS and analyzed for radioactivity in a Beckman γ-spectrometer. Non-specific binding was determined in the presence of 1 µM unlabeled CRF. Receptor concentration and affinity were estimated by computer analysis of the binding data (5). Protein concentration was measured by the method of Lowry et al. (6) using BSA as standard.

Stimulation of ACTH release by CRF peptides was analyzed in anterior pituitary cells prepared by trypsin digestion (7) and cultured in suspension for 48 h. (8), then transferred to fresh medium 199 containing 0.2% BSA and aprotinin 100 KIU/ml. One ml aliquots of the cell suspension containing 1 to 2 x 10^5 cells were incubated with increased concentrations of CRF peptides in polyethylene vials at 37°C under 5% CO_2, 95% O_2. After 3 h, cells were separated by centrifugation at 1500 x g and ACTH release was measured by radioimmunoassay of the incubation medium (3). The concentrations of the peptides producing a half-maximal ACTH response (EC_{50}) were determined by computer analysis, using a four-parameter logistic function (9).

RESULTS

The rate and extent of ^{125}I-Tyr-CRF binding to anterior pituitary membranes were highly temperature-dependent, as shown in Figure 1A. At 22°C, specific binding reached equilibrium at 30 min and remained at a plateau for about 60 min. When the temperature was increased to 37°C,



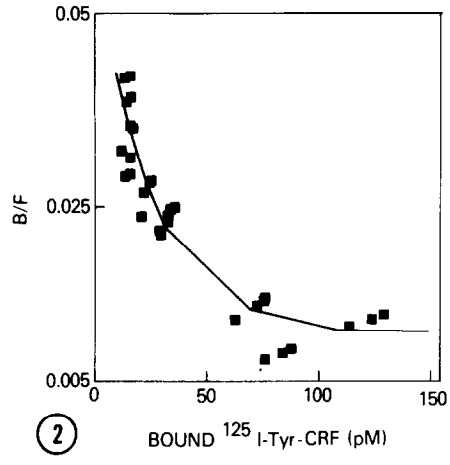


Figure 1. A. Time and temperature dependence of ^{125}I-Tyr-CRF binding to pituitary particles. B. Dissociation of ^{125}I-Tyr-CRF from pituitary particles: After equilibration at 22°C, 1 µM of unlabeled CRF was added and incubations were continued for up to 3 h. Data points are the mean of duplicate incubations in a representative experiment.

Figure 2. Scatchard analysis of ^{125}I-Tyr-CRF binding to pituitary particles, based on pooled data from five experiments.

the rate of association was more rapid but the period of equilibrium became shorter. A marked decrease in the rate of association was observed during incubation at 16 and 4°C. Addition of unlabeled CRF after binding had reached equilibrium resulted in rapid dissociation of the bound radioactivity, with a half-time of 2.5 min at 37°C and 30 min at 22°C (Figure 1B). A linear increase in the binding of the labeled ligand was observed with increasing amounts of particulate protein up to 500 µg (not shown). Under conditions in which 8% of the added radioactivity was bound to receptors, non-specific binding was 1 to 1.5% of the total.

The binding of ^{125}I-Tyr-CRF to pituitary particles was saturable and on Scatchard analysis (Figure 2) showed a high-affinity component with dissociation constant (Kd) of 0.76 $\pm$ 0.23 x 10^{-9} M and concentration of 47.4 $\pm$ 11.8 fmol/mg. In most experiments, a second class of low-affinity sites with Kd of 10^{-6} M was also detected.

The ligand specificity of the pituitary binding sites for ^{125}I-Tyr-CRF is shown in Figure 3, which illustrates the inhibition of tracer binding

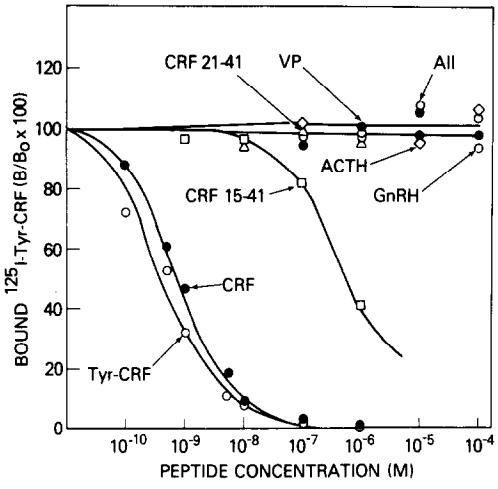
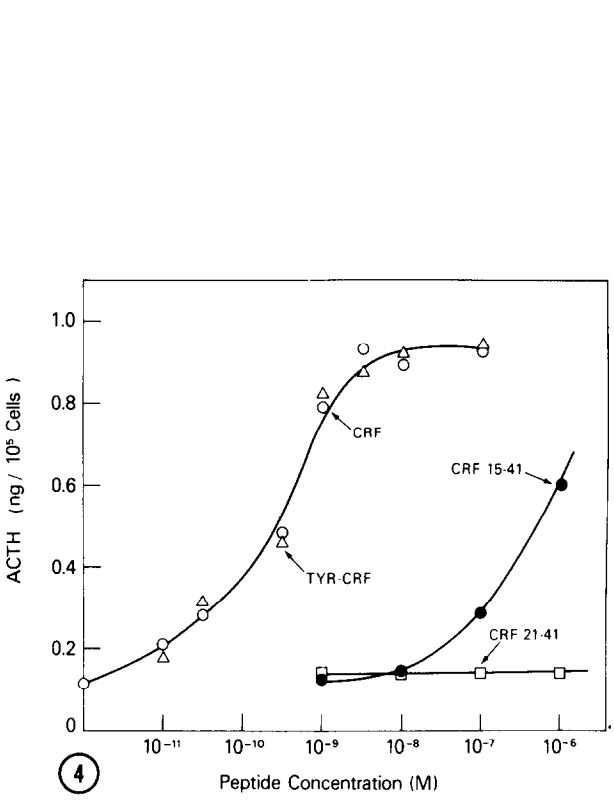


Figure 3. Ligand specificity of the pituitary binding sites for CRF. Each point is the mean of duplicate incubations in a representative experiment, performed by addition of increasing concentrations of CRF and other peptides to pituitary particles incubated with 10^5 cpm of ^{125}I-Tyr-CRF as described in Materials and Methods.

by CRF peptides and non-related peptides. The native CRF peptide and Tyr-CRF were equipotent, with half-maximum inhibitory concentrations (IC_{50}) of 4.0 and 7.0 x 10^{-10} M, respectively. The active CRF 15-41 fragment displayed ^{125}I-Tyr-CRF with an IC_{50} of 5.6 x 10^{-7} M, while the inactive CRF 21-41 sequence was without effect. Unrelated peptides including ACTH, angiotensin II, vasopressin, and GnRH, had no binding-inhibition activity at concentrations up to 10^{-4} M.

The biological significance of the CRF binding sites in pituitary particles was indicated by comparison of the binding activities of the different CRF peptides with their ACTH-releasing potencies in isolated pituitary cells (Figure 4). Both CRF and Tyr-CRF stimulated ACTH release by a factor of 8-fold, with half-maximum effective concentrations (EC_{50}) of 3.2 and 2.8 x 10^{-10} M, respectively. Consistent with previous observations (3), the 15-41 CRF fragment was a weak agonist with full intrinsic activity (EC_{50} 5 x 10^{-7} M) and the 21-41 fragment was completely inactive. In each experiment, the activities of the peptides upon stimulation of ACTH release were similar to their relative binding affinities.



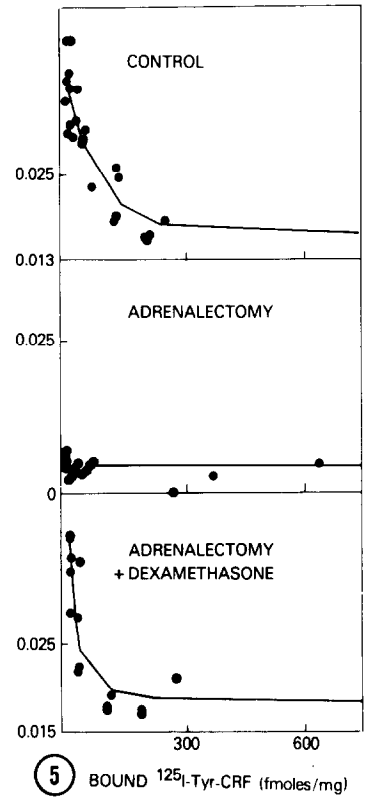


Figure 4. Dose-response curves for the stimulation of ACTH release from dispersed pituitary cells incubated with CRF, CRF 15-41 and CRF 21-41.

Figure 5. Effects of adrenalectomy and steroid replacement on pituitary CRF receptors. Binding-inhibition studies were performed on pituitary particles prepared from groups of 10 rats, and the pooled data from Scatchard analyses of 2 to 4 individual groups are shown. (A) Sham-operated controls (n=4). (B) Rats killed 4 days (n=3) and 6 days (n=1) after adrenalectomy. (C) Rats killed 4 days after adrenalectomy plus daily injection of 50 µg dexamethasone (n=2).

To determine whether pituitary CRF receptors undergo regulatory changes during variations in ACTH secretion, CRF binding sites were measured in pituitary particles from adrenalectomized rats 4 to 6 days after surgery. As shown in Figure 5, the concentration of high-affinity CRF receptor sites was markedly decreased after adrenalectomy, when only the low affinity sites were detectable. When corticosteroid replacement with dexamethasone (50 µg/day) was given, the loss of high-affinity sites was largely prevented, with retention of 70% of the original receptors (21 vs 36 fmol/mg).

DISCUSSION

These studies with radioiodinated Tyr-CRF have identified specific binding sites for the hypothalamic peptide in the anterior pituitary gland. The validity of this peptide analog for receptor binding analysis was indicated by the demonstration that the addition of Tyr at the amino terminus of the molecule does not alter the biological activity of CRF. In most of the experiments, CRF binding to pituitary particles was resolved into 2 components. The significance of the second site is uncertain, but its low binding affinity renders it unlikely to be a true receptor site.

The high-affinity CRF binding site has the properties of a physiologically relevant receptor, being saturable and of limited capacity, with high specificity for CRF and its active analogs. Also, the Kd of the CRF receptor (0.8 nM) is in accord with the concentration of the hypothalamic peptide (0.1 nM) in rat hypophysial portal blood (10). In addition, there was a close similarity between the potencies of the different CRF analogues to stimulate ACTH release in pituitary cells and their relative binding-inhibition potencies. The biological significance of the CRF receptor was also supported by the changes in high-affinity sites observed during altered rates of ACTH secretion, after adrenalectomy and dexamethasone treatment. Thus, when the secretion of ACTH, and presumably of CRF, was increased by adrenalectomy, pituitary CRF receptors were markedly decreased. This change was prevented by steroid replacement treatment, which would reduce CRF release as well as the elevated ACTH secretion caused by the absence of corticosteroid feedback after adrenalectomy. Although receptor occupancy by endogenous CRF could contribute to the receptor loss observed in adrenalectomized rats, the rapid dissociation of the labeled hormone during binding studies *in vitro* renders it improbable that occupancy would account for the marked decrease in CRF receptors. It is more likely that CRF receptors are down-regulated by elevated levels of endogenous CRF, comparable to the homologous regulation of other peptide hormone receptors including insulin (11), GH (12), LH (13), and vascular angiotensin II receptors (14).

These studies with ^{125}I-Tyr-CRF have identified high-affinity CRF binding sites in the anterior pituitary gland that represent the specific receptors responsible for mediating the actions of CRF on ACTH release, and have shown that CRF receptors are regulated by changes in the concentration of hypothalamic CRF to which they are exposed.

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