Identification and Characterization of a Pituitary Corticotropin-releasing Factor Binding Protein by Chemical Cross-linking

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A corticotropin-releasing factor (CRF) binding protein has been identified based on the chemical cross-linking of ovine [Nle18, m-Tyr26]CRF (125I-oCRF) to bioassay and pituitary membranes using disuccinimidyl suberate (DSS). The apparent molecular weight of the cross-linked complex determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography was approximately 75,000 and was slightly decreased in its nonreduced state, suggesting the presence of intramolecular disulfide bonds. Subtracting the molecular weight of 125I-oCRF, the binding protein appeared to have a molecular weight of ~70,000. The cross-linking was specific since an excess (1 μM) of an unrelated peptide (insulin) did not affect the appearance of the M, 75,000 band.

The concentration of CRF required to inhibit cross-linking by 50% was found to be similar to that determined for bovine pituitary CRF receptors by radioreceptor assay. The nonhydrolyzable GTP analogue 5'-guanylylimidodiphosphate dose dependently inhibited the cross-linking of 125I-oCRF to the M, 70,000 protein. 50 nM of the inactive CRF analogue, [Ala14]oCRF, had no effect on the cross-linking, an observation which is consistent with this compound's low potencies in CRF receptor assays. The nonhydrolyzable GTP analogue 5'-guanylylimidodiphosphate did not affect the appearance of the M, 70,000 band. Subtracting the molecular weight of 125I-oCRF, the binding protein appeared to have a molecular weight of ~70,000. The cross-linking was specific since an excess (1 μM) of an unrelated peptide (insulin) did not affect the appearance of the M, 75,000 band.

Corticotropin-releasing factor (CRF),¹ a 41-amino acid hy-

potalamic peptide, stimulates the secretion of ACTH and the other peptides encoded by the pro-opiomelanocortin (POMC) gene (1, 2) as well as POMC gene transcription in anterior pituitary cells (3, 4). The actions of CRF are believed to be initiated by its binding to specific receptors which have been identified in the rat (5, 6) and bovine (7) anterior pituitary. These binding sites are of high affinity, having a reported Kd of approximately 1 nM measured in both bovine and rat anterior pituitary membranes (7).

CRF receptors appear to be linked to the adenylate cyclase system since it has been demonstrated that binding of CRF to its receptors activates adenylate cyclase (8), resulting in increased levels of intracellular cAMP (9–11). In addition, CRF binding can be affected by divalent cations, as well as by guanyl nucleotides (7). These findings further suggest the involvement of the adenylate cyclase system since GTP binding regulatory proteins are thought to couple the receptor to the adenylate cyclase (12, 13).

In order to investigate the molecular properties of the CRF receptor, we have used the bifunctional cross-linking agent disuccinimidyl suberate (DSS) to identify and characterize CRF binding proteins present in bovine anterior pituitary membranes. In this report we describe a M, 70,000 protein which specifically binds radiolabeled CRF and shows binding characteristics similar to those of high affinity CRF receptors.

**EXPERIMENTAL PROCEDURES**

**Materials—**[Nle18, Tyr26]oCRF and [Ala14]oCRF were synthesized by J. Rivier (The Salk Institute) using solid phase methods (14). Ni2+ (low pH) was purchased from Du Pont–New England Nuclear. DTT and Gpp(NH)p were from Sigma. DSS was obtained from Pierce Chemical Co. All reagents required for SPS-PAGE were purchased from Bio-Rad as was the protein kit used for protein determinations.

**Membrane Preparation—**Plasma membranes were prepared from bovine pituitaries as previously described (7). Briefly, anterior pituitaries were homogenized in 10 volumes of 0.32 M sucrose and centrifuged for 5 min at 600 × g. The supernatant was collected and centrifuged at 40,000 × g for 30 min and the pink upper layer of the resulting pellet was resuspended in 0.32 M sucrose at a protein concentration of 60-80 mg/ml. This preparation (P2) was further purified on a discontinuous density gradient consisting of 15 ml each of 28 and 47% sucrose, and then centrifuged at 100,000 × g for 60 min. The resulting pellet was resuspended with 0.32 M sucrose to give 60–80 mg of protein/ml (P3). All procedures were carried out at 4 °C. Each experiment was conducted with a different membrane preparation.

**Cross-linking—**Ovine [Nle18, Tyr26]CRF (oCRF) was iodinated as described previously (7) except that ovalbumin was used in place of bovine serum albumin (BSA) and that the purified radiolabeled peptide was stored in 60% acetonitrile with 0.1% trifluoroacetic acid in H2O in the absence of BSA.

Two-milligram aliquots of bovine pituitary membrane preparation (P2) diluted in binding buffer (10 mM HEPES, pH 7.6, 10% sucrose, 5 mM MgSO4, 2 mM EGTA, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride) were incubated with 1 nM 125I-oCRF (∼2 × 106 cpm) with or without the addition of unlabeled oCRF as indicated in the figure legend. The total assay volume was 0.8 ml. After a 90-min period, binding was terminated with the addition of 10 ml of ice-cold HEPES buffer and centrifuged at 40,000 × g for 20 min. The radio- labeled membrane pellet, resuspended in 5 ml of HEPES buffer, was cross-linked by the addition of 0.5 mM DSS for 20 min. The reaction was quenched with 30 ml of 100 mM Tris-HCl, pH 7.4, followed by centrifugation at 40,000 × g for 20 min. The pellet was solubilized

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¹ The abbreviations used are: CRF, corticotropin-releasing factor; ACTH, corticotropin; Nle, norleucine; "125I-oCRF, ovine [Nle18, m-Tyr26]CRF; oCRF, ovine [Nle18, Tyr26]CRF, DSS, disuccinimidyl suberate; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; Gpp(NH)p, 5'-guanylylimidodiphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, (ethylenbis(2- aminoethyl)enitrile)tetraacetic acid; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.
with 100 μl of SDS sample buffer (3% SDS in 80 mM Tris-HCl, pH 6.8) with or without 10 mM dithiothreitol (DTT) as indicated in the figures. Each experiment was conducted a minimum of two times.

SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed essentially as described by Laemmli (15). Samples were heated to 100 °C for 3 min and applied to 7.5% acrylamide slab gels. [3H]Methylated proteins (Amersham Corp.) served as molecular markers. Following electrophoresis the gels were fixed with 50% methanol, 10% acetic acid for 1 h, dried, placed in apposition to x-ray film with intensifying screens, and stored at −70 °C for 4–14 days. The relative intensities of bands were determined by scanning densitometry.

Receptor Binding Assay—Binding of 125I-oCRF to bovine plasma membranes (P2g) was measured according to Perrin et al. (7). In brief, 30 μl aliquots of P2g, diluted in assay buffer (50 mM Tris-HCl, pH 7.4, 0.1% BSA, 10% sucrose, 5 mM MgSO4, 2 mM EGTA, 1% Trasylol, and 0.2 mM phenylmethylsulfonyl fluoride) to give 500–600 μg of protein, were incubated with 100,000 cpm of 125I-oCRF (120 pm) in the presence of increasing concentrations of unlabeled oCRF for 90 min at 22 °C. The final assay volume was 200 μl. Unbound trace was washed from the membranes by the addition of 2 ml of ice-cold 10 mM DTT and subjected to 125I-filtration through Whatman GF/C filters presoaked in 1% RSA. The filters were counted in a γ-counter (Searle) at 85% efficiency.

RESULTS AND DISCUSSION

Ovine [Nle10, m.125I-Tyr28]CRF (125I-oCRF) was used in this study as a probe to identify CRF binding proteins in the pituitary. This radiolabeled ligand binds specifically to high affinity CRF receptors located in bovine and rat pituitary membranes (7). In addition, physiological doses of this ligand stimulate ACTH release from cultured rat anterior pituitary cells (7). Thus, 125I-oCRF should serve as a suitable ligand for the identification of putative CRF receptor proteins by chemical cross-linking methods.

Following SDS-PAGE of bovine anterior pituitary membranes, which were covalently cross-linked to 125I-oCRF, several radiolabeled species with approximate molecular weights of 190,000, 95,000, 75,000, and 45,000 were observed on the corresponding autoradiogram (Fig. 1). The M, 75,000 band demonstrated specificity for CRF in that its appearance could be inhibited by the addition of unlabeled oCRF. Although the appearance of the minor M, 95,000 band was also blocked by oCRF, this was not consistently observed (5 of 33 autoradiograms demonstrated a band at the M, 95,000 position). Furthermore, by scanning densitometry, the intensity of the 95,000 band was found to be less than 8% of that of the 75,000 band. The addition of proteolytic enzyme inhibitors (1% Trasylol, 2 mM phenylmethylsulfonyl fluoride, and 0.1% bacitracin) during the preparation of the membranes did not alter this pattern of labeling. Though the possibility of the protein represented by this 95,000 band as being a specific CRF
**CRF Receptor Cross-linking**

**Fig. 3.** Effect of Gpp(NH)p on cross-linking of \(^{125}\)I-oCRF to bovine pituitary membranes. Binding of \(^{125}\)I-oCRF to membranes was carried out in the absence or presence of the nonhydrolyzable GTP analogue, Gpp(NH)p. Cross-linking with 0.5 mM DSS and subsequent SDS-PAGE were performed as described under "Experimental Procedures." Samples were solubilized in SDS buffer under reducing (+) and nonreducing (-) condition.

**Fig. 4.** Effect of the inactive CRF analogue [Ala\(^{14}\)]oCRF on the cross-linking of \(^{125}\)I-oCRF to bovine anterior pituitary membranes. Binding of \(^{125}\)I-oCRF to bovine anterior pituitary membranes was carried out in the absence (lane 1) or presence (lane 2) of 50 nM oCRF or 50 nM of the inactive CRF analogue [Ala\(^{14}\)]oCRF (lane 3). Cross-linking with 0.5 mM DSS and SDS-PAGE were conducted as described under "Experimental Procedures." The samples were reduced with 10 mM DTT prior to electrophoresis.

**Fig. 5.** Cross-linking of \(^{125}\)I-oCRF to BSA. 0.02% BSA in cross-linking buffer was incubated for 90 min with \(^{125}\)I-oCRF under conditions identical to those used for cross-linking to membrane preparations. DSS was added to give a final concentration of 0.5 mM. After 20 min, an equal volume of 2 X sample buffer (+) or (-) DTT was introduced to quench the reaction. SDS-PAGE was conducted as described under "Experimental Procedures."

The apparent electrophoretic mobility of this M, 70,000 protein was increased in its nonreduced form (Fig. 1) which is indicative of the presence of intramolecular disulfide bonds. This has been reported of other receptors for peptide hormones such as insulin (16, 17) and epidermal growth factor (18) which have subsequently been shown by molecular cloning to have a high cysteine content within their extracellular domains.

To examine the binding characteristics of this M, 70,000 CRF binding protein, a competition curve was constructed by the addition of increasing concentrations of unlabeled oCRF during the binding reaction (Fig. 2). By measuring the relative densities of the corresponding M, 75,000 band by scanning densitometry, the concentration of oCRF required for 50% inhibition (IC\(_{50}\)) was found to be approximately 0.2 nM (Fig. 2A). This value was very close to the IC\(_{50}\) determined by radioreceptor assay using bovine anterior pituitary membranes (0.4 nM). The cross-linking of \(^{125}\)I-oCRF to the M, 70,000 protein was specific in that an excess amount of an unrelated peptide, 1 \(\mu\)M insulin, was unable to inhibit the cross-linking.

Membrane-associated guanine nucleotide binding proteins (G proteins) are believed to act as signal transducers by coupling receptors to effectors (12, 13). In the CRF receptor system, binding of the hormone to the pituitary receptors activates adenylate cyclase (the effector) through a mechanism involving divalent cations and GTP (7-11). As a means of comparing the M, 70,000 binding protein to the CRF receptor, the effects of the nonhydrolyzable GTP analogue, Gpp(NH)p, on the cross-linking were examined. The appearance of the M, 75,000 band could be dose dependently inhibited by the addition of Gpp(NH)p during the binding reaction (Fig. 3), indicating that this CRF binding protein is associated with a G protein in the manner of a hormone receptor. This finding is consistent with the previous report that micromolar doses of Gpp(NH)p can reduce the number of measurable specific high affinity CRF binding sites in bovine anterior pituitary membrane preparations (7).
Pharmacologically, this \( M_{r} \), 70,000 CRF binding protein was found to resemble the CRF receptor in that 50 nM of the inactive analogue [Ala\(^{14}\)]-oCRF (7) did not displace the cross-linking of \( ^{125}\text{I}\)-oCRF (Fig. 4). This agrees with the relative binding and biological potencies of this CRF derivative (7).

In a previous report, a \( M_{r} \), 75,000 growth hormone-releasing factor receptor was identified on the basis of cross-linking studies (19). More recently, however, Zysk et al. (20) have shown that this \( M_{r} \), 75,000 complex could only be observed in the presence of bovine serum albumin (\( M_{r} \), 67,000–69,000) and so have suggested that this putative growth hormone-releasing factor receptor was, in fact, BSA. In view of this potential problem, the present study was conducted without the addition of BSA. Although there exists a possibility of the presence of endogenous BSA in the membrane preparation, the characteristics of the cross-linking (specificity, affinity, and sensitivity to guanine nucleotides) strongly argues against the \( M_{r} \), 70,000 CRF binding protein identified here as being BSA. As a further control, 0.02% BSA was cross-linked to \( ^{125}\text{I}\)-oCRF (Fig. 5), generating a pattern of radiolabeled bands, \( M_{r} \), 69,000 (nonreduced) and 80,000 (reduced), that does not at all resemble the pattern of the bands observed following cross-linking to bovine pituitary membranes (compare with Fig. 1). The bands produced by cross-linking to BSA are much sharper and there is a far greater difference in apparent molecular weights between the reduced and nonreduced states. Finally, cross-linking to BSA could not be blocked by the addition of increasing concentrations of unlabeled oCRF (Fig. 5). These distinctions clearly indicate that the \( M_{r} \), 75,000 band observed in Figs. 1–4 cannot be due to endogenous albumin, if at all present.

In conclusion, we have identified a \( M_{r} \), 70,000 protein from bovine anterior pituitary plasma membranes which specifically binds oCRF with a high affinity. The binding characteristics of this protein closely resemble those of the pituitary CRF receptor in terms of its affinity for the CRF ligand and for an inactive CRF derivative, as well as its sensitivity to a GTP analogue. Furthermore, the apparent presence of intramolecular disulfide bonds in this CRF binding protein and its association with G proteins are properties attributed to hormone receptors. In preliminary studies, CRF affinity chromatography of solubilized bovine pituitary membranes have resulted in the purification of a \( M_{r} \), ~70,000 CRF binding protein as observed on silver-stained, 7.5% SDS-polyacrylamide gels. Together, these observations strongly suggest that the \( M_{r} \), 70,000 protein represents the biological CRF receptor.

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REFERENCES


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