The Brain Corticotropin-releasing Factor (CRF) Receptor Is of Lower Apparent Molecular Weight Than the CRF Receptor in Anterior Pituitary

EVIDENCE FROM CHEMICAL CROSS-LINKING STUDIES*

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The ligand binding subunits of the corticotropin-releasing factor (CRF) receptors in brain and anterior pituitary of a number of species have been identified by chemical affinity cross-linking using the homobifunctional cross-linking agent disuccinimidyl suberate and 125I-Tyr5-oCRF (ovine CRF). In homogenates of rat, monkey, and human cerebral cortex, 125I-Tyr5-oCRF was covalently incorporated into a protein of M_r = 58,000. Under identical conditions in the anterior pituitary of rat, monkey, cow, and pig, 125I-Tyr5-oCRF was incorporated into a protein of apparent M_r = 75,000. The specificity of the labeling was typical of the CRF binding site since both the cerebral cortex and pituitary-labeled proteins exhibited the appropriate pharmacological rank order profile characteristic of the CRF receptor (Ne21-Tyr6-25-oCRF ≈ rat/human CRF ≈ ovine CRF ≈ a-helical CRF9-41 > a-helical oCRF(9-41) ≥ oCRF(7-41) ≥ rat/human CRF(1-20) ≈ vasoactive intestinal peptide). In addition to the major labeled proteins, 125I-Tyr5-oCRF was incorporated into higher molecular weight peptides which may represent precursors and into lower molecular weight components which may represent fragments of the major labeled proteins or altered forms of the CRF binding subunit. In summary, these data indicate a heterogeneity between brain and pituitary CRF receptors with the ligand binding subunit of the brain CRF receptor residing on a M_r = 58,000 protein, while in the anterior pituitary, the identical binding subunit resides on a protein of apparent M_r = 75,000.

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1 Postdoctoral Fellow of the Medical Research Council of Canada.
2 The abbreviations used are: CRF, corticotropin-releasing factor; oCRF, ovine CRF; DSS, disuccinimidyl suberate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitri1)tetraacetic acid. receptors have been identified, characterized, and localized in rat (6-8), bovine (7, 9), monkey (10), and human (11) pituitary gland, as well as in rat (8, 12-17), monkey (8, 18), and human (19) brain using a variety of stable iodine-125-labeled analogs of ovine or rat/human CRF. While data from receptor binding (7, 12-14, 16), adenylate cyclase (16, 17), and in vitro and in vitro pharmacologic (20-22) studies suggest that the structural requirements and pharmacology for CRF activity are shared by brain and pituitary receptors, there is evidence demonstrating differential regulation of CRF receptors in rat pituitary and brain following a variety of experimental manipulations including adrenalectomy (15, 16), glucocorticoid administration (24), and stress (25). Very recently, CRF binding proteins have been identified using chemical cross-linking of radiiodinated analogs of CRF to bovine anterior pituitary membranes (26) and AtT-20 mouse pituitary tumor cells (27) with molecular weights of approximately 70,000 and 66,000, respectively. However, the CRF binding protein remains to be identified and characterized in brain. In the present study, we have used 125I-Tyr5-oCRF and the homobifunctional cross-linking reagent disuccinimidyl suberate (DSS) to characterize the ligand binding subunit of brain and pituitary CRF receptors. We report the initial identification of a brain CRF binding protein which is of a lower apparent molecular weight than the pituitary CRF receptor in various species including rat, cow, pig, monkey, and human.

EXPERIMENTAL PROCEDURES

Materials and Tissues---125I-Tyr5-oCRF (specific activity, 2200 Ci/mmol) was purchased from Du Pont-New England Nucleon (Boston, MA). Unlabeled ovine and rat/human CRF were purchased from Peninsula Laboratories (Belmont, CA). All other CRF analogs and fragments were synthesized and generously provided by Dr. Jean Rivier of the Salk Institute. Standard reagents were obtained from either Sigma or Bio-Rad. DSS was purchased from Pierce Chemical Co. Bovine and porcine pituitaries were purchased from Pel-Freeze, rhesus monkey pituitaries were kindly provided by Dr. Thomas R. Insel of the National Institute of Mental Health, and human postmortem cerebral cortex was kindly provided by Dr. Robert Struble of the Brain Research Center at Johns Hopkins University School of Medicine. Male Sprague-Dawley rats (Harlan Industries) were killed by decapitation, and various brain regions and anterior pituitary were dissected on ice and frozen at -70 °C.

Methods---125I-Tyr5-oCRF Binding to Membrane Receptors—Tissues (approximately 150 mg) were weighed and homogenized in 7 ml of Dulbecco's phosphate-buffered saline, 10 mM MgCl2, 2 mM EGTA, 0.15% bovine serum albumin, 0.15 mM bacitracin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.0015% aprotinin, pH 7.0, at 22 °C using a Brinkmann Polytron at a setting of 5 for 20 s. Membranes were sedimented by centrifugation at 40,000 × g for 10 min at 4 °C, resuspended in the same buffer (assay buffer), and centrifuged again at 40,000 × g for 10 min at 4 °C. The final pellet was resuspended in...
assay buffer to a working concentration of 20–40 mg original wet weight/ml and kept on ice until use. 125I-Tyr2-oCRF binding was performed as previously described (14) using a final concentration of 100–200 pm 125I-Tyr2-oCRF and 1 μM unlabeled rat/human CRF to define the non-specific binding. Protein determinations were performed according to the method of Lowry et al. (28) using bovine serum albumin as the standard.

Chemical Cross-linking of 125I-Tyr2-oCRF to Receptors—The procedure for chemical cross-linking of 125I-Tyr2-oCRF to membrane homogenates followed closely that of Pilch and Czech (29) with some modification. DSS was dissolved in dimethyl sulfoxide to yield a final concentration of 1.5 mM in the assay tubes. Following the 2-h incubation, 10 μl of the DSS solution was added to each tube and allowed to react for 20 min at 22 °C. The reaction was terminated by the addition of 1.0 ml of ice-cold 10 mM Tris-HCl and 1 mM EDTA, pH 7.0, at 0–4 °C (wash buffer) and centrifugation at 12,000 rpm for 5 min in a Beckman Microfuge. The pellets were washed gently by the addition of 1.0 ml of ice-cold wash buffer and recentrifuged. Final pellets were monitored for bound radioactivity in a γ counter (LKB) at 80% efficiency and then solubilized in electrophoresis sample buffer (see below) before being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE—Samples for electrophoresis were resuspended in SDS-PAGE sample buffer containing 50 mM Tris-HCl, 10% glycerol, 5% β-mercaptoethanol, and 0.005% bromphenol blue (pH 6.8 at 22 °C) and incubated for 45 min at 22 °C. Samples were then transferred to a boiling water bath and incubated for a further 15 min before being electrophoresed on a discontinuous slab gel (6% stacking and 12% running in most studies and 4% stacking and 7.5% running in order to resolve higher molecular weight differences) according to the method of Laemmli (30). An equivalent amount of protein (200–300 μg) was loaded onto each lane and typically run overnight at a constant current of 5–15 mA. Prestained protein standards (Sigma) were included on each gel and used to calculate a standard curve from their relative mobilities. Following electrophoresis, gels were dried (Bio-Rad slab gel dryer) and exposed to Kodak X-AR film using a film developing system (Amersham). In conjunction with the gels used for autoradiography, duplicate gels were run concomitantly, and each lane was cut in 2-mm slices and monitored for radioactivity.

RESULTS AND DISCUSSION

125I-Tyr2-ovine CRF binding in homogenates of rat anterior pituitary and frontal cerebral cortex was saturable and of high affinity with apparent dissociation constant (Kd) values of 110 and 170 pm, respectively, and maximum concentration of binding sites (Bmax) of 25 and 13 fmol/mg of protein, respectively (data not shown). These values are comparable to those previously reported in brain (12–15, 18, 19) and anterior pituitary (6, 7, 9, 10).

In rat frontal cerebral cortex, olfactory bulb, and cerebellar homogenates, 125I-Tyr2-oCRF was functionally cross-linked to a membrane protein with an apparent Mr = 58,000 with the specificity (as defined by displacement by 1 μM rat/human CRF) of the CRF receptor (Figs. 1 and 2, A, C, and D). In all brain areas examined, specific labeling was also observed in a protein of apparent molecular weight of approximately 116,000; however, incorporation of radiolabel into the Mr = 116,000 component in brain represented less than 30% of the total 1 μM rat/human CRF-displaceable covalent binding (Fig. 2). Autoradiograms of 125I-Tyr2-oCRF cross-linked to brain membranes, with longer periods of exposure, also showed faint labeling of a protein fragment at Mr ~ 20,000–22,000, albeit with a much lower intensity. A somewhat different profile was obtained in the rat anterior pituitary. In anterior pituitary membranes, covalently bound 125I-Tyr2-oCRF followed by SDS-PAGE and autoradiography revealed a broad and diffuse pattern of labeling for the higher molecular weight components. In order to resolve the diffuse labeling observed in the anterior pituitary into specifically labeled protein components, gels were sliced and counted and aliquots were simultaneously run on 7.5% and 12% acrylamide gels (SDS-PAGE). DSS was dissolved in dimethyl sulfoxide to yield a final concentration of 1.5 mM in the assay tubes. Following the 2-h incubation, 10 μl of the DSS solution was added to each tube and allowed to react for 20 min at 22 °C. The reaction was terminated by the addition of 1.0 ml of ice-cold 10 mM Tris-HCl and 1 mM EDTA, pH 7.0, at 0–4 °C (wash buffer) and centrifugation at 12,000 rpm for 5 min in a Beckman Microfuge. The pellets were washed gently by the addition of 1.0 ml of ice-cold wash buffer and recentrifuged. Final pellets were monitored for bound radioactivity in a γ counter (LKB) at 80% efficiency and then solubilized in electrophoresis sample buffer (see below) before being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE—Samples for electrophoresis were resuspended in SDS-PAGE sample buffer containing 50 mM Tris-HCl, 10% glycerol, 5% β-mercaptoethanol, and 0.005% bromphenol blue (pH 6.8 at 22 °C) and incubated for 45 min at 22 °C. Samples were then transferred to a boiling water bath and incubated for a further 15 min before being electrophoresed on a discontinuous slab gel (6% stacking and 12% running in most studies and 4% stacking and 7.5% running in order to resolve higher molecular weight differences) according to the method of Laemmli (30). An equivalent amount of protein (200–300 μg) was loaded onto each lane and typically run overnight at a constant current of 5–15 mA. Prestained protein standards (Sigma) were included on each gel and used to calculate a standard curve from their relative mobilities. Following electrophoresis, gels were dried (Bio-Rad slab gel dryer) and exposed to Kodak X-AR film using a film developing system (Amersham). In conjunction with the gels used for autoradiography, duplicate gels were run concomitantly, and each lane was cut in 2-mm slices and monitored for radioactivity.

RESULTS AND DISCUSSION

125I-Tyr2-ovine CRF binding in homogenates of rat anterior pituitary and frontal cerebral cortex was saturable and of high affinity with apparent dissociation constant (Kd) values of 110 and 170 pm, respectively, and maximum concentration of binding sites (Bmax) of 25 and 13 fmol/mg of protein, respectively (data not shown). These values are comparable to those previously reported in brain (12–15, 18, 19) and anterior pituitary (6, 7, 9, 10). Although complete displacement of 125I-Tyr2-oCRF incorporation by 1 μM rat/human CRF was observed in the Mr = 75,000 component (60% of total specific labeling), while minor specific incorporation was present in the Mr = 100,000–130,000 (20%), 24,000 (12%), and 20,000 (8%) membrane components; a very minor fragment (<1%) was observed at Mr = 44,000 (refer to Fig. 1). Although complete displacement of 125I-Tyr2-oCRF incorporation in 1 μM rat/human CRF was observed in the Mr = 100,000–130,000, 75,000, and 20,000 proteins, incorporation into the Mr = 24,000 component was only partially inhibited (Fig. 2B). As also shown in Fig. 1, in the absence of the homobifunctional cross-linking reagent DSS, 125I-Tyr2-oCRF did not covalently incorporate into any membrane components. Molecular weights of prestained markers are indicated on the left.

While the apparent Mr = 75,000 band corresponds to the CRF binding protein previously described by Nishimura et al. (26) in bovine anterior pituitary, it is unclear whether the minor, specifically labeled, Mr = 20,000 band represents a functional subunit of the CRF receptor in the pituitary or whether it is a degradation product containing the binding site for CRF. Alternatively, since some of the smaller peptides (comparable Mr values of 20,000–22,000) migrate with the same mobility in both tissues, they may prove to be a common link between the Mr = 58,000 protein in brain and the apparent Mr = 75,000 protein in pituitary. Furthermore, it is unclear whether the Mr = 100,000–130,000 membrane component in anterior pituitary and the Mr = 116,000 component in brain represents a common precursor(s) to the respective CRF binding proteins. It is unlikely, however, that the Mr = 58,000 component...
CRF binding protein in brain is a breakdown product of the apparent \( M_r = 75,000 \) binding site observed in pituitary since we could not demonstrate any incorporation whatsoever at apparent \( M_r = 75,000 \) in any of the brain tissues examined (refer to Figs. 1 and 2). The differences in molecular weight observed for CRF binding sites in brain and anterior pituitary may relate to the extent of glycosylation in the two tissues or to the expression of different mRNAs. Further study is needed in order to elucidate these possibilities.

The incorporation of \(^{125}\text{I}-\text{Tyr}^2\text{-oCRF} \) into the major protein band in rat anterior pituitary was greater when compared with the brain regions examined with 65%, 50%, and 30% of the relative values in the anterior pituitary present in olfactory bulb, frontal cortex, and cerebellum, respectively (Figs. 1 and 2). The increased incorporation of \(^{125}\text{I}-\text{Tyr}^2\text{-oCRF} \) in the pituitary reflects the higher density of receptors in the pituitary than in the brain since the affinity of the receptors, under conditions identical with those used in the covalent cross-linking studies, are comparable in the two tissues (see above). Furthermore, the relative density of the receptors in the four tissues is in agreement with previous homologate binding studies demonstrating higher concentrations of CRF receptors in the anterior pituitary than in the olfactory bulb which has a higher density than in the frontal cortex and cerebellum (14, 17).

In order to establish the pharmacological specificity of the apparent \( M_r = 75,000 \) protein in rat anterior pituitary and the \( M_r = 58,000 \) protein in the rat frontal cortex, cross-linking studies were performed in the presence of various CRF-related and -unrelated fragments and analogs. The rank order profile of the CRF-related peptides to inhibit binding at the \( M_r = 58,000 \) protein in the frontal cortex and the apparent \( M_r = 75,000 \) and \( M_r = 20,000 \) proteins in the pituitary was identical (Fig. 3). At a 100 nM concentration of each of the peptides, the pharmacological profile was as follows: Nle\(^{21}\), Tyr\(^{32}\) - oCRF ≈ rat/human CRF ≈ ovine CRF ≈ \( \alpha \)-helical CRF(6-41) > \( \alpha \)-helical oCRF(9-41) ≈ oCRF(7-41) > rat/human CRF(1-20) ≈ vasoactive intestinal peptide. This pharmacological profile is comparable to that demonstrated for the CRF binding site in homogenates of rat anterior pituitary (7, 9), and rat brain (12-15), and the pharmacological profile of the various peptides in stimulating adenylate cyclase activity in brain (16, 17). Furthermore, the respective protein bands in the frontal cortex (\( M_r = 58,000 \)) and rat anterior pituitary (apparent \( M_r = 75,000 \)) exhibited a pharmacological specificity for CRF analogs and fragments that correlates extremely well with their relative intrinsic potency in stimulating or inhibiting anterior pituitary secretion of adrenocorticotropic hormone secretion in vitro (1, 2, 31, 32). The specificity in both tissues is further strengthened by the lack of inhibitory activity of fragments (rat/human CRF(1-20)) and vasoactive intestinal peptide. These data strongly suggest that the \( M_r = 58,000 \) protein in brain and the \( M_r = 75,000 \) protein in anterior pituitary represent the CRF binding protein in the respective tissues and further substantiate earlier suggestions that some structural requirements for CRF activity are shared by brain and pituitary receptors (7, 12-14, 16, 17, 20-22).

Previous studies have identified and characterized CRF receptors in rat (6-8), bovine (7, 9), monkey (10), and human (11) anterior pituitary and in rat (8, 12-17), monkey (8, 18),
Pharmacologic specificity and rank order of potencies of various CRF-related and unrelated peptides. Rat frontal cortex (A) and anterior pituitary (B) membranes were incubated with $^{125}$I-Tyr-oCRF alone or in the presence of various peptides and allowed to cross-link in the presence of DSS. Total and nonspecific binding (defined in the presence of 1 pM rat/human CRF) in both tissues (lanes 1 and 2 in both tissues), defined the specific labeled binding sites in the frontal cortex ($M_r = 58,000$) and apparent $M_r = 75,000$ in the pituitary (apparent $M_r = 75,000$). The optical density values corresponding to the $M_r = 58,000$ protein in cerebral cortex (A) and apparent $M_r = 75,000$ protein in anterior pituitary (B) are presented below each respective autoradiogram. In both tissues, the rank order of potencies of the peptides shown above was identical using a 100 nM concentra-

Cerebral cortex (CTX) preparations of human (lanes 1 and 2), monkey (lanes 3 and 4), and rat (lanes 7 and 8) all showed identical labeling of a protein with an apparent molecular weight of 58,000. The anterior pituitary (ANT PIT) membrane preparations of monkey (lanes 3 and 6), rat (lanes 9 and 10), pig (lanes 11 and 12), and cow (lanes 13 and 14) all showed the same labeling pattern with the binding site residing on the major specifically labeled protein with an apparent molecular weight of 75,000. Nonspecific labeling in each case was defined in the presence of 1 μM unlabeled rat/human CRF and is shown in lanes 2, 4, 6, 8, 10, 12, and 14. The patterns shown are typical of two or three experiments. Molecular weight standards are indicated on the left.

Fig. 3. Pharmacologic specificity and rank order of potencies of various CRF-related and unrelated peptides. Rat frontal cortex (A) and anterior pituitary (B) membranes were incubated with $^{125}$I-Tyr-oCRF alone or in the presence of various peptides and allowed to cross-link in the presence of DSS. Total and nonspecific binding (defined in the presence of 1 μM rat/human CRF) in both tissues (lanes 1 and 2 in both tissues), defined the specific labeled binding sites in the frontal cortex ($M_r = 58,000$) and in the pituitary (apparent $M_r = 75,000$). The optical density values corresponding to the $M_r = 58,000$ protein in cerebral cortex (A) and apparent $M_r = 75,000$ protein in anterior pituitary (B) are presented below each respective autoradiogram. In both tissues, the rank order of potencies of the peptides shown above was identical using a 100 nM concentra-

and human (19) brain that have comparable kinetic and pharmacologic characteristics. In order to determine whether the heterogeneity in the molecular weight of rat brain and anterior pituitary CRF receptors was unique to the species, we identified the CRF binding protein by chemical cross-linking in the cerebral cortex and anterior pituitary of a variety of species including human, rhesus monkey, cow, and pig. As shown in Fig. 4, the heterogeneity of CRF receptors in brain and pituitary demonstrated in rat was consistent across the species examined. Specifically, rhesus monkey, porcine, and bovine pituitaries all showed comparable profiles on SDS-PAGE with respect to incorporation of $^{125}$I-Tyr-oCRF into proteins that were identical with the profile described above for rat anterior pituitary (see Figs. 1 and 2B). Similarly, the SDS-PAGE profiles of cerebral cortex in human, monkey, and rat were identical, with incorporation of $^{125}$I-Tyr-oCRF into a single major protein band of $M_r = 58,000$. Thus, using chemical cross-linking techniques, we have identified a brain CRF binding protein with a molecular weight different from the CRF binding protein in the pituitary. While the pharmacological characteristics and structure-activity relationships of the CRF binding sites in brain and pituitary appear to be similar (see above), there is evidence in the literature demonstrating that brain and pituitary receptors are differentially regulated. For example, adrenalectomy (15, 23), glucocorticoid administration (24), and certain types of stress (25) have been shown to down-regulate CRF receptors in the rat anterior pituitary without affecting CRF bind-

Fig. 4. $^{125}$I-Tyr-oCRF cross-linking patterns of cerebral cortex and anterior pituitary membranes of different species. Cerebral cortex (CTX) preparations of human (lanes 1 and 2), monkey (lanes 3 and 4), and rat (lanes 7 and 8) all showed identical labeling of a protein with an apparent molecular weight of 58,000. The anterior pituitary (ANT PIT) membrane preparations of monkey (lanes 3 and 6), rat (lanes 9 and 10), pig (lanes 11 and 12), and cow (lanes 13 and 14) all showed the same labeling pattern with the binding site residing on the major specifically labeled protein with an apparent molecular weight of 75,000. Nonspecific labeling in each case was defined in the presence of 1 μM unlabeled rat/human CRF and is shown in lanes 2, 4, 6, 8, 10, 12, and 14. The patterns shown are typical of two or three experiments. Molecular weight standards are indicated on the left.

The experiment was repeated once with identical results.
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...ing sites in brain. In other studies, alterations in CRF receptors have been demonstrated in the cerebral cortex in Alzheimer's disease (19) and in atropine-treated rats (33). At present, it is unclear whether the differential regulation of brain and pituitary receptors may relate in part to the differences in the apparent molecular weights of the binding subunits that we have identified in the present study.

In summary, we have identified and characterized brain and pituitary CRF binding proteins with identical pharmacological characteristics but different apparent molecular weights (brain, Mₐ = 58,000; anterior pituitary, Mₐ = 75,000). The differences in the apparent molecular weights of the brain and pituitary CRF receptors observed in rats following a variety of endocrine manipulations including adrenalectomy, glucocorticoid administration, and stress. The physiological relevance of the differences in the molecular weights remains to be elucidated.

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