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Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain

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ABSTRACT **The present study reports the isolation of a cDNA clone that encodes a second member of the corticotropin-releasing factor (CRF) receptor family, designated as the CRF_2 receptor. The cDNA was identified using oligonucleotides of degenerate sequence in a PCR paradigm. A PCR fragment obtained from rat brain was utilized to isolate a full-length cDNA from a rat hypothalamus cDNA library that encoded a 411-amino acid protein with ≈70% identity to the known CRF_1 receptor over the entire coding region. When expressed in mouse Ltk^- cells, this receptor stimulates cAMP production in response to CRF and known CRF-like agonists. CRF and the nonmammalian CRF-related peptides sauvagine and urotensin I stimulate adenylate cyclase activity in a dose-dependent manner with a rank order of potency different from that of the CRF_1 receptor: sauvagine > urotensin ≥ rat/human CRF > ovine CRF. Tissue distribution analysis of the mRNAs by reverse transcriptase–PCR shows CRF_2 receptor mRNA is present in rat brain and detectable in lung and heart. *In situ* hybridization studies indicate specific expression within the brain in the ventromedial nuclei of the hypothalamus, the lateral septum, the amygdala, and entorhinal cortex, but there is unremarkable expression in the pituitary. An additional splice variant of the CRF_2 receptor with a different N-terminal domain has been identified by PCR, encoding a putative protein of 431 amino acids. Thus, the data demonstrate the presence of another functional CRF receptor, with significant differences in the pharmacological profile and tissue distribution from the CRF_1 receptor, which would predict important functional differences between the two receptors.**

Corticotropin-releasing factor (CRF), a 41-amino acid peptide, regulates the secretion of adrenocorticotropin and other proopiomelanocortin products from the anterior pituitary. CRF also coordinates the endocrine, behavioral, and autonomic responses to stress. Within the past few years, substantial evidence has accumulated from both laboratory and clinical studies implicating CRF as a physiological mediator of stress responses and stress-induced disorders (1–6). Immunocytochemical studies have shown that CRF is found within the paraventricular nucleus of the hypothalamus as well as limbic areas such as the central and medial nuclei of the amygdala, the bed nucleus of the stria terminalis, substantia inominata, septum, preoptic area, the lateral hypothalamus, and brain stem nuclei involved in stress responses and regulation of autonomic function, such as the locus coeruleus, the parabrachial nucleus, and the dorsal vagal complex (see ref. 7). CRF, when administered intracerebroventricularly, results in behavioral, physiological, and autonomic responses that are similar to those observed when animals are exposed to a stressful environment (4–6).

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CRF mediates its action through high-affinity membrane receptors that have been extensively characterized by radioligand binding studies in brain and pituitary (for review, see ref. 8). A receptor for CRF has recently been cloned and functionally expressed by three groups (9–12). This receptor, designated as CRF_1, is a member of a distinct family of seven transmembrane domain receptors, which includes receptors for calcitonin, vasoactive intestinal peptide (VIP), parathyroid hormone, and others (13). The receptor is potently activated by CRF and sauvagine, the related frog skin peptide, to stimulate adenylate cyclase. Its mRNA is predominantly expressed in cerebellum, pituitary, cerebral cortex, and olfactory bulb, which is consistent with the primary sites of action of CRF.

While there is a general correspondence of the distribution of ^{125}I-labeled CRF binding sites and CRF_1 receptor mRNA in brain with the functional actions of CRF, there are several brain areas with some apparent discrepancies. For example, there is a generally low level of expression and uniform distribution of ^{125}I-labeled CRF binding sites and CRF_1 receptor mRNA in the hypothalamus, whereas CRF has robust *in vitro* and *in vivo* effects in hypothalamus on neuroendocrine, autonomic, and behavioral parameters (4, 5, 8, 14, 15). Similarly, CRF has potent electrophysiological and behavioral effects in the locus coeruleus while the distribution of ^{125}I-labeled CRF binding sites and CRF_1 receptor mRNA is not notable in this brainstem nucleus (4, 5, 8, 14). This and the observation that CRF exerts multiple peripheral functions led us to hypothesize the existence of other CRF receptor subtypes that could account for such effects. Accordingly, we designed a degenerate PCR experiment based on the known human, rat, and mouse sequences of the CRF_1 receptor. Here we present data showing the existence of a second CRF receptor (designated CRF_2 receptor)§ isolated from rat brain and describe experiments addressing its functional characterization and tissue localization.

MATERIALS AND METHODS

PCR Cloning and Sequence Analysis. Poly(A)$^+$ RNA was isolated from whole rat brain (excluding cerebellum) and lung by using PolyATract mRNA isolation kits (Promega). First-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega), which was subsequently dG-tailed by terminal deoxynucleotidyltransferase (Promega). This procedure adds a homopolymer tail of ≈18 deoxyguanine nucleotides at the 3′-OH of the first-strand cDNA. Synthetic oligonucleotides of degenerate sequence were designed based on segments of transmembrane domain 3 and transmembrane

Abbreviations: CRF, corticotropin-releasing factor; r/hCRF, rat/human CRF; oCRF, ovine CRF; VIP, vasoactive intestinal peptide; ORF, open reading frame.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U16235).

domain 7 of the CRF_1 receptor (Met-Phe-Gly-Glu-Gly-Cys-Tyr and Tyr-Cys-Phe-Leu-Asn-Ser-Glu, respectively). Nucleotide sequences of primers (5′ to 3′ with N = A,T,G, or C; Y = T or C; R = A or G) were as follows: domain 3, AGCACGAGCTCATGTYGGNGARGGNTGYTA; domain 7, AGCACGAATTCYTCNCTRTTNARRAARCARTA. The first round of PCR amplification [30 cycles at 94°C for 1 min, 52°C for 2 min, and 72°C for 2 min with a Hybaid (Middlesex, U.K.) model Omnigene thermal cycler] used the transmembrane domain 7 oligonucleotide in combination with a $d(C)_{18}$ oligonucleotide [5′-ACTGTAAGCTT$(C)_{18}$-3′], where the dG-tailed rat brain cDNA was used as a template. The products of this reaction served as template for a second round of amplification with the transmembrane domain 3- and the transmembrane domain 7-derived oligonucleotides. The products of this reaction were size-selected between 400 and 500 bp, digested with restriction enzymes *Sac* I and *Eco*RI, and ligated into pBluescript (Stratagene). Isolated clones were sequenced by the dideoxynucleotide chain-termination method (16) with Sequenase (United States Biochemical). Sequence data were analyzed with DNASTAR sequence software (DNAstar, Madison, WI).

Cell Transfection. The CRF_2 receptor cDNA was subcloned into the expression vector pCDM7-Amp. Mouse Ltk^- cells were transfected with a standard DEAE-dextran protocol. Briefly, Ltk^- cells were plated at 3×10^6 cells per 10-cm dish 24 h prior to the experiment. Transfections were performed in 3 ml of Dulbecco's modified Eagle's medium (DMEM) containing DEAE-dextran (Sigma; 100 μg/ml), 100 μM chloroquine (Sigma), and 10 μg of DNA for 4 h. Subsequently the cells were grown in DMEM containing 10% (vol/vol) fetal bovine serum for 24 h, replated in 24-well plates, and grown for an additional 24 h.

cAMP Accumulation. cAMP accumulation was assayed essentially as described (17) with modifications to adapt the assay to whole cell preparations. The standard assay mixture contained the following components in a final volume of 0.5 ml: 2 mM L-glutamine, 20 mM Hepes, and 1 mM isobutylmethylxanthine in DMEM. In stimulation studies, whole cells containing the transiently transfected CRF_2 receptor or stably transfected CRF_2 receptor were incubated for 1 h at 37°C with various concentrations of CRF-related and unrelated peptides. After the incubation, the medium was aspirated, and the cells were rinsed once gently with fresh medium. Next, 300 μl of 95% ethanol/20 mM HCl (EtOH/HCl) was added to each well and incubated at −20°C for 16–18 h. The solutions were removed to 1.5-ml Eppendorf tubes, and the wells were washed with an additional 200 μl of EtOH/HCl, which was pooled with the first fraction. The samples were lyophilized and resuspended with 500 μl of 0.05 M sodium acetate (pH 6.2). The measurement of cAMP in the samples was performed using a radioimmunoassay kit from Biomedical Technologies (Stoughton, MA).

***In Situ* Hybridization.** Rats were sacrificed by decapitation, and brains and pituitaries were removed and frozen in liquid isopentane (−42°C). Subsequently, tissues were sectioned (15 μm) on a cryostat maintained at −20°C and thaw-mounted onto polylysine-coated microscope slides. Sections were stored at −80°C prior to tissue fixation. To fix, sections were placed directly into buffered 4% (wt/vol) paraformaldehyde at room temperature. After 60 min, slides were rinsed in isotonic phosphate-buffered saline (10 min) and treated with proteinase K [1 μg/ml in 100 mM Tris·HCl (pH 8.0)] for 10 min at 37°C. Subsequently, sections underwent successive washes in water (1 min), 0.1 M triethanolamine, pH 8.0/0.25% acetic anhydride (for 10 min), and 2× SSC (0.3 mM NaCl/0.03 mM sodium citrate, pH 7.2; for 5 min). Sections were then dehydrated through a graded alcohol series and air-dried.

RNA probes were synthesized using Maxiscipt RNA transcription kits (Ambion, Austin, TX). Post-fixed sections were hybridized with 1.0×10^6 dpm of ^{35}S-labeled UTP-labeled RNA probes in hybridization buffer [75% (vol/vol) formamide/10% (wt/vol) dextran sulfate/3× SSC/50 mM sodium phosphate, pH 7.4/1× Denhardt's solution/yeast tRNA (0.1 mg/ml)/10 mM dithiothreitol] in a total volume of 25 μl. The diluted probe was applied to sections on a glass coverslip. Sections were hybridized overnight at 55°C in a humid environment.

After the hybridization, sections were washed in 2× SSC for 5 min and then treated with RNase A (200 μg/ml in 10 mM Tris·HCl, pH 8.0/0.5 M NaCl) for 60 min at 37°C. Subsequently, sections were washed in 2× SSC for 5 min, 1× SSC for 5 min, 0.5× SSC for 60 min at hybridization temperature, 0.5× SSC at room temperature for 5 min, then dehydrated in a graded alcohol series, and air-dried. For signal detection, sections were dipped in Amersham LM-1 emulsion and stored dessicated in light-tight boxes for 6 weeks at 4°C.

RESULTS AND DISCUSSION

Cloning Strategy. To determine whether other receptors exist that are related to the cloned CRF_1 receptor (9–12), a degenerate anchor-PCR experiment was designed. The procedure of using nested degenerate oligonucleotides has been used to amplify less-abundant cDNAs (18–20). However, this procedure typically requires the design of at least three degenerate oligonucleotides. The first two oligonucleotides are used to create an enriched "template" for the second round of PCR, which then requires an additional oligonucleotide corresponding to target DNA created in the first round. Each new oligonucleotide requires the presence of a corresponding sequence in the target gene and, therefore, increases the risk of missing related genes. To reduce this risk, an anchor-PCR was combined with nested degenerate oligonucleotides. This procedure reduces the number of necessary degenerate oligonucleotides to two, thus increasing the probability of identifying sequences related to the initial template design (in this case the CRF receptor).

Initially, a single degenerate antisense oligonucleotide (corresponding to a portion of transmembrane domain 7) in combination with a poly(dC) oligonucleotide was used to selectively amplify from first-strand rat brain dG-tailed cDNA. Next, oligonucleotide primers of degenerate sequence corresponding to an amino acid sequence in the putative transmembrane domain 3 from the known CRF receptor was used in combination with the original degenerate oligonucleotide to amplify DNA fragments from the first reaction. Amplification products were size-selected, subcloned, and sequenced. In one experiment, 24 products were subcloned, 8 of which were picked (based on size) for sequencing. Seven of these matched the known sequence for the cloned rat CRF receptor (9, 12). One of clones (CRD23) exhibited a similar, but distinct, sequence from the others. Analysis of this clone showed that it encoded an open reading frame (ORF) for a protein ≈80% identical to the rat CRF receptor.

Isolation of Full-Length Receptors. To rapidly identify a full-length sequence for this apparently distinct receptor, two approaches were taken. The first approach was to use the original CRD23 PCR fragment as a probe to screen a rat hypothalamus cDNA library. The second was to use an anchor-PCR strategy to quickly assess the sequence of the 5′ end of the cDNA. Screening of the library resulted in the isolation of five clones, two of which encoded what appeared to be the entire ORFs. One of those turned out to have an unspliced intron inserted in the message. The other encoded the entire ORF of the cDNA except for 20 bp in front of the stop codon. The two clones were combined to yield a cDNA with a 1233-bp ORF encoding a predicted protein of 411 amino acids, which is designated as the CRF_2 receptor (Fig. 1). The protein exhibits seven putative hydrophobic regions corresponding to

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GCGGCCCCTCATCTCCGTGAGCCCCGAGGCTTCTCTTGGCCAAGGTCCTAGGAGTGATCC-155
GATTGAGAGCGGCGCCCCAAAGCTGCCGGGCTGGCCGGGGTGGGCGGGGAGGCACCTGGA -95
CGCTGCACTCTCTGGTGGCTCCGCGTCGCGCCAGGTCCCTCGCAGCCACGCGGGGCGCGC -35
ACTCCCACTCCCAACGCGCGCGGCTCCGGAGCGCAATGGACGCGGCGCTGCTCCTCAGCC  25
                                        M  D  A  A  L  L  L  S    8
TGCTGGAGGCCAACTGCAGCCTGGCACTGGCCGAAGAGCTGCTTTTGGACGGCTGGGGAG  85
L  L  E  A  N  C  S  L  A  L  A  E  E  L  L  L  D  G  W  G     28
AGCCCCCGGACCCCGAAGGTCCCTACTCCTACTGCAACACGACCTTGGACCAGATCGGGA 145
E  P  P  D  P  E* G  P  Y  S  Y  C  N  T  T  L  D  Q  I  G     48
CCTGCTGGCCCCAGAGCGCGCCTGGAGCCCTAGTGGAGAGACCATGCCCCGAATACTTCA 205
T  C  W  P  Q  S  A  P  G  A  L  V  E  R  P  C  P  E  Y  F     68
ACGGCATCAAGTACAACACGACCCGGAATGCCTACAGAGAATGCCTGGAGAATGGGACCT 265
N  G  I  K  Y  N  T  T  R  N  A  Y  R  E  C  L  E  N  G  T     88
GGGCCTCAAGGATCAACTACTCACACTGTGAACCCATTTTGGATGACAAGCAGAGGAAGT 325
W  A  S  R  I  N  Y  S  H  C  E  P  I  L  D  D  K  Q  R  K    108
ATGACCTGCATTACCGAATCGCCCTCATCATCAACTACCTGGGCCACTGTGTTTCCGTGG 385
Y  D  L  H  Y  R  I  A  L  I  I  N  Y  L  G  H  C  V  S  V    128
TGGCCCTGGTGGCTGCTTTCCTGCTTTTCCTAGTGCTGCGGAGTATCCGCTGCCTGCGGA 445
V  A  L  V  A  A  F  L  L  F  L  V  L  R  S  I  R  C  L  R    148
ATGTGATCCACTGGAACCTCATCACCACCTTCATCCTGAGAAACATCACGTGGTTCCTGC 505
N  V  I  H  W  N  L  I  T  T  F  I  L  R  N  I  T  W  F  L    168
TGCAACTCATCGACCACGAAGTGCATGAGGGCAATGAGGTCTGGTGCCGCTGCGTCACCA 565
L  Q  L  I  D  H  E  V  H  E  G  N  E  V  W  C  R  C  V  T    188
CCATATTCAACTACTTTGTGGTCACCAACTTCTTCTGGATGTTTGTGGAAGGCTGCTACC 625
T  I  F  N  Y  F  V  V  T  N  F  F  W  M  F  V  E  G  C  Y    208
TGCACAGGCCATCGTCATGACGTACTCCACGGAGCATCTGCGCAAGTGGCTCTTCCTCT 685
L  H  T  A  I  V  M  T  Y  S  T  E  H  L  R  K  W  L  F  L    228
TCATTGGATGGTGCATACCCTGCCCTATCATTGTCGCCTGGGCAGTTGGCAAACTCTACT 745
F  I  G  W  C  I  P  C  P  I  I  V  A  W  A  V  G  K  L  Y    248
ATGAGAATGAGCAGTGCTGGTTTGGCAAGGAACCTGGTGACTTAGTGGACTACATCTACC 805
Y  E  N  E  Q  C  W  F  G  K  E  P  G  D  L  V  D  Y  I  Y    268
AGGGCCCCATCATCCTCGTGCTCCTCATCAATTTTGTGTTTCTGTTCAACATCGTCAGGA 865
Q  G  P  I  I  L  V  L  L  I  N  F  V  F  L  F  N  I  V  R    288
TCCTGATGACAAAACTGCGAGCCTCCACCACATCCGAGACCATCCAGTACAGGAAGGCAG 925
I  L  M  T  K  L  R  A  S  T  T  S  E  T  I  Q  Y  R  K  A    308
TGAAGGCCACCCTGGTCCTCCTCCCCCTGTTGGGCATCACCTACATGCTCTTCTTTGTCA 985
V  K  A  T  L  V  L  L  P  L  L  G  I  T  Y  M  L  F  F  V    328
ATCCTGGAGAGGACGACCTGTCACAGATTGTGTTCATCTACTTCAACTCTTTCCTGCAGT1045
N  P  G  E  D  D  L  S  Q  I  V  F  I  Y  F  N  S  F  L  Q    348
CCTTTCAGGGTTTCTTTGTGTCCGTTTTCTACTGCTTCTTCAATGGAGAGGTGCGCTCCG1105
S  F  Q  G  F  F  V  S  V  F  Y  C  F  F  N  G  E  V  R  S    368
CCCTGAGAAAGCGGTGGCACCGTTGGCAGGACCACCACGCCCTCCGAGTGCCTGTGGCCC1165
A  L  R  K  R  W  H  R  W  Q  D  H  H  A  L  R  V  P  V  A    388
GGGCCATGTCCATTCCCACATCGCCCACCAGGATCAGCTTCCACAGCATCAAGCAGACAG1225
R  A  M  S  I  P  T  S  P  T  R  I  S  F  H  S  I  K  Q  T    408
CTGCCGTGTGATCCCCTGTCACCCATCTGCCCAGCACTCCACCACCGAGGCGGCTTCCTC1285
A  A  V                                                       411
ATTCTTCACAGCCTTCCCTGGGTCCTCCTTGCTACACTGACCCTTGGGTGCAGGAGAAGG1345
GGGGGTGGATGAACTCTCCTGCCGGAAGAAAGGAAAACTATGAAATGGAGGCTCTGAAAG1405
ACCAGG                                                        1410
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FIG. 1. Nucleotide and translated amino acid sequences of the CRF_2 receptor. Underlined amino acids indicate putative transmembrane domains. Boxed amino acids indicate potential N-linked glycosylation sites. An asterisk indicates the region of sequence difference between $CRF_{2\alpha}$ and $CRF_{2\beta}$. The $CRF_{2\beta}$ has the 5′ amino acid sequence of MGHPGSLPSAQLLLCLYSLLPLLQVAQPGRPLQDQPLWTLLEQYCHRTTTRNFS, which replaces the $CRF_{2\alpha}$ 5′ end up to the asterisk.

putative transmembrane domains, five potential N-linked glycosylation sites, and six Cys residues in the N-terminal extracellular domain. When compared to other receptors of the neuropeptide family, this CRF_2 receptor exhibits a remarkable 71% overall identity with the known CRF_1 receptor (Fig. 2). It shows ≈30% homology with other members of this neuropeptide receptor superfamily.

In the anchor-PCR approach, an oligonucleotide specific to CRF_2 was used in a paradigm similar to that described above for degenerate oligonucleotides except dG-tailed lung cDNA was included as template, in addition to dG-tailed brain cDNA. The sequence of some of the fragments isolated from brain were identical to the hypothalamus cDNA clone. However, others from both brain and lung cDNAs were identical only from amino acid 35 onward. The 5′ end of these clones had the first 34 amino acids replaced with an ORF of 54 amino acids, including a translational start codon. We have tentatively named the two apparent splice forms of the receptor $CRF_{2\alpha}$ and $CRF_{2\beta}$ for the hypothalamus and brain/lung forms, respectively. The $CRF_{2\beta}$ form encodes a putative protein of 431 amino acids and the 5′ sequence is indicated in Fig. 1. Chen *et al.* (10) found, in their human cDNA for CRF receptor, two apparent splice forms of the same receptor, which they named CRF-R_1 and CRF-R_2. Their CRF-R_1 form appears to be the predominant form and no species homolog or tissue specificity has been identified for the CRF-R_2 form. We propose that

	CRF_1	CRF_2	GHRH	GLUC	SECR	VIP	PTH	PACAP	CALC
CRF_1	100								
CRF_2	**71.0**	100							
GHRH	33.0	30.0	100						
GLUC	30.0	28.0	33.0	100					
SECR	32.0	31.0	39.0	38.0	100				
VIP	31.0	31.0	43.0	37.0	46.0	100			
PTH	29.0	30.0	33.0	32.0	38.0	35.0	100		
PACAP	30.0	29.0	43.0	41.0	50.0	55.0	37.0	100	
CALC	32.0	35.0	26.0	27.0	26.0	27.0	31.0	28.0	100

FIG. 2. Homology table. This table represents the overall sequence identity among peptide transmitter receptors in the "gut–brain" peptide receptor family. Amino acid sequences were analyzed by the Jotun–Hein method (DNAstar). All comparisons are based on the rat species homologs. GHRH, growth hormone releasing hormone; GLUC, glucagon; SECR, secretin; PTH, parathyroid hormone; PACAP, pituitary adenylate cyclase activating peptide; CALC, calcitonin.

receptor splice forms from the same gene should maintain the same numerical designation but be distinguished by Greek letter designations. Thus, CRF-R_1 and CRF-R_2 should be redesignated as $CRF_{1\alpha}$ and $CRF_{1\beta}$ receptors. The $CRF_{2\alpha}$ and $CRF_{2\beta}$ splice variants identified in the present study indicate two forms of the second known receptor subtype. The pharmacology or tissue-specific distribution of the $CRF_{2\beta}$ receptor has yet to be determined; however, preliminary PCR experiments with $CRF_{2\beta}$-specific primers indicate expression in brain and lung. The work presented below focuses strictly on the

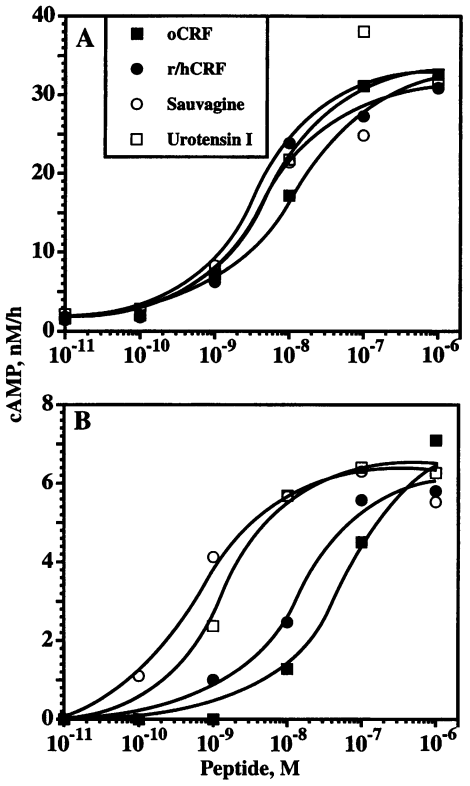

FIG. 3. Stimulation of cAMP production in cells stably transfected with CRF_1 receptor cDNA (*A*) and transiently transfected with CRF_2 receptor cDNA (*B*). Transfected cells were incubated with increasing concentrations of various CRF-related peptides and assessed for the amount of cAMP produced at 37°C. Each concentration was determined in duplicate and the data are representative of three determinations.

hypothalamus clone ($CRF_{2\alpha}$ receptor) since the $CRF_{2\beta}$ form was cloned only by PCR.

Second Messenger Coupling and Pharmacological Analysis. The CRF_1 receptor has been cloned (9–12) and shown to be linked to the stimulation of adenylate cyclase. Since the CRF_2 receptor shows striking similarity to the CRF_1 receptor, particularly in the third intracellular domain, it was presumed to share this functional linkage. To confirm that the CRF_2 receptor couples to the stimulation of adenylate cyclase, the receptor cDNA was subcloned into an expression vector (pCDM7-Amp) and transiently transfected into mouse Ltk^- cells. Cells transfected with CRF_2 responded in a dose-dependent manner to rat/human CRF (r/hCRF), ovine CRF (oCRF), and the CRF-related peptides sauvagine and urotensin I (Fig. 3*B*) with half-maximal efficacy (EC_{50}) values of ≈20 nM, 80 nM, 0.5 nM, and 2 nM, respectively. In Ltk^- cells stably overexpressing the CRF_1 receptor, r/hCRF, oCRF, sauvagine, and urotensin were nearly equipotent at stimulating cAMP accumulation (Fig. 3*A*) with EC_{50} values of ≈4 nM, 10 nM, 3 nM, and 4 nM, respectively. Furthermore, the unrelated peptides VIP, vasopressin, and growth hormone-releasing hormone did not stimulate any cAMP production in these cells. In addition, the biologically weak deamidated form of CRF was 100- to 1000-fold less potent than the native form, confirming the structural requirements for specificity at both receptors. Transient transfection of the CRF_1 receptor in Ltk^- cells yielded essentially identical results to the stable transfections. The maximal stimulation of cAMP accumulation induced through the CRF_1 receptor was greater than the CRF_2 receptor (Fig. 3) presumably due to the higher density of receptors in stably vs. transiently transfected cells, respectively. This is supported by the finding that transient expression of the CRF_1 receptor also results in a lower maximal cAMP response (data not shown). The putative CRF receptor antagonists α-helical oCRF-(9–41) (21) and [D-Phe^{12},Nle^{21,38},Ala^{32}]hCRF-(12–41) were able to competitively antagonize 10 nM r/hCRF- or sauvagine-stimulated cAMP accumulation with an approximately equal affinity of 100 nM in cells transfected with CRF_1 or CRF_2 receptors (data not shown).

CRF_1 and CRF_2 receptors clearly have different pharmacological profiles with respect to the CRF-related peptides. The most noteable difference is the lower potency of CRF for the CRF_2 receptor. Predictably, preliminary data in a direct binding assay with radioiodinated CRF did not show a high level of specific binding to the CRF_2 receptor (data not shown). This may be due to the inability to achieve a sufficient concentration of radiolabeled ligand to bind to the CRF_2 receptor in equilibrium binding assays. However, preliminary receptor crosslinking studies with the CRF_2 receptor and ^{125}I-labeled oCRF do demonstrate receptor labeling, albeit with apparent low incorporation (data not shown).

An intriguing aspect of the CRF_2 receptor is the finding that sauvagine and urotensin I are more potent activators than CRF itself. Sauvagine and urotensin I are CRF-like peptides that have been isolated from frog and fish, respectively (22, 23). Sauvagine has been shown to be equipotent with CRF at the CRF_1 receptor (9), and urotensin I has been shown to have a higher affinity for the CRF-binding protein than CRF or

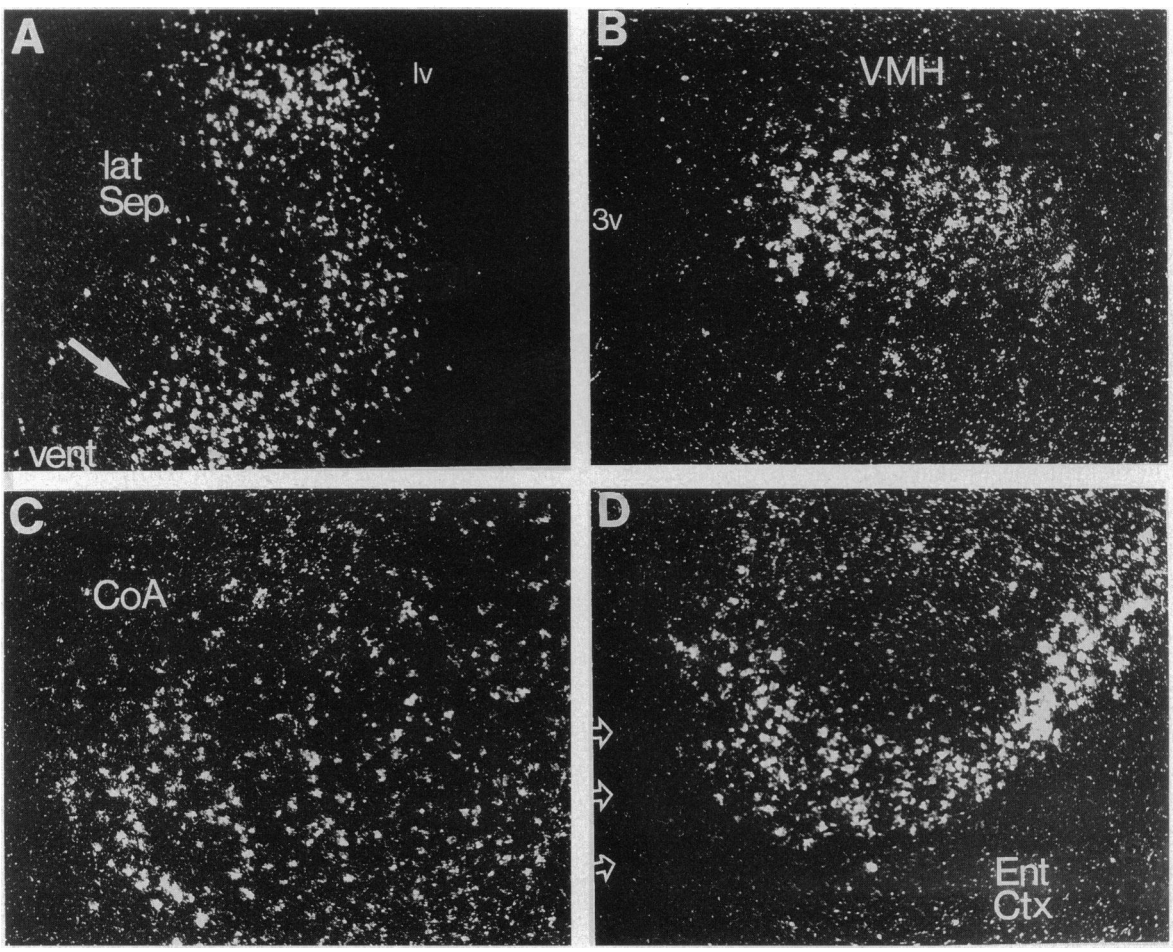

FIG. 4. Dark-field photomicrographs of cells hybridized with a ^{35}S-labeled complementary RNA CRF_2 probe in lateral septum (*A*), ventromedial hypothalamic nucleus (VMH) (*B*), cortico-amygdaloid nucleus (*C*), and entorhinal cortex (*D*). In *A*, note the high density of silver grains in the ventral aspect (vent) of the nucleus (arrow). In *D*, prominent hybridization signal was detected in deeper cortical layers; arrows indicate the section edge. lv, Lateral ventricle; 3v, third ventricle.

sauvagine (24). In addition, sauvagine and urotensin I have been reported to have behavioral and autonomic effects that are more potent than CRF itself (25), suggesting that these actions may be mediated in part by CRF_2 receptors. Since CRF itself is also found in fish (22), urotensin I or sauvagine may have, as of yet, undiscovered mammalian counterparts. If this is true, then CRF_2 may be the primary target of such molecules. Until a mammalian counterpart of sauvagine or urotensin I is identified, it is reasonable to classify CRF_2 receptor as a CRF-like receptor, since it does bind CRF with high affinity and is located in CRF-responsive areas.

Tissue Distribution. CRF_2 receptor mRNA was below the level of detection by Northern blot analysis in all tissues examined [poly(A)$^+$ RNA from brain, heart, spleen, lung, liver, skeletal muscle, kidney, and testis at 2 μg per lane]. However, by reverse transcriptase–PCR, we were able to detect receptor message in the brain, lung, kidney, and heart. Since PCR is extremely sensitive and difficult to quantify, *in situ* hybridization histochemistry was used to localize CRF_2 receptor mRNA expression. An antisense RNA probe complementary to the CRF_2 receptor was synthesized ($\approx$66% homology to CRF_1 receptor). The specificity of the hybridization signal was established by confirming a lack of hybridization in sections treated with a sense-strand RNA probe and sections pretreated with RNase A prior to hybridization with antisense RNA probe. By using the antisense CRF_2 RNA probe, significant levels of hybridization were evident in many brain regions. Prominent expression was evident in lateral septum, the ventromedial nucleus of the hypothalamus, several amygdaloid nuclei, and the entorhinal cortex (Fig. 4). CRF_2 receptor mRNA expression was undetectable in neocortex and cerebellar cortex, in contrast to the high levels of CRF_1 receptor expression in these regions (14). Similarly, CRF_2 receptor expression was unremarkable in pituitary lobes where CRF_1 receptor expression is readily detectable (14).

This heterogeneous distribution of CRF receptor subtypes is likely to reflect distinct functional roles for CRF_2 receptors vs. CRF_1 receptors within the brain. Of the areas with high expression levels of CRF_2 mRNA, evidence from lesion and electrode stimulation studies suggests that the ventromedial hypothalamic nucleus is critical for food intake regulation (26), whereas the lateral septum, amygdala, and entorhinal cortex are intimately involved in limbic circuitry associated with determining the affective state.

There are several lines of preclinical and clinical evidence that support a role for brain CRF in neuropsychiatric, neurodegenerative, metabolic, and feeding disorders (see refs. 1 and 27). Briefly, the data suggest that CRF may be hypersecreted in brain in major depression, anxiety-related disorders, and anorexia nervosa. In contrast, deficits in brain CRF may contribute to dementia associated with neurodegenerative disorders such as Alzheimer disease. Furthermore, hypofunction of hypothalamic CRF has been implicated in the development of obesity syndromes. The identification of CRF_2 receptors will now allow a more precise definition of the CRF functional pathways and provide the basis for the development of selective research tools and/or therapeutics specifically targeted toward these systems.

SUMMARY

The data presented in this study describe a gene that encodes a member of the CRF receptor family (CRF_2 receptor). Analysis of the CRF_2 receptor has revealed several interesting aspects of CRF physiology. (*i*) The CRF_2 receptor exhibits a distinct pharmacological profile from the CRF_1 receptor. (*ii*) The distribution of CRF_2 receptor mRNA shows a unique highly localized distribution in the brain, with prominant expression in the limbic regions. This is in contrast to CRF_1 receptor (14) or the CRF-binding protein (28), which are both widely and somewhat codistributed throughout the brain, particularly in cerebral cortex and in pituitary. In accordance with these findings, it can be hypothesized that different aspects of CRF physiology may be altered by drugs displaying selectivity for CRF_1 vs. CRF_2 receptors. It is tempting to speculate that the two receptor subtypes will mediate different aspects of CRF function in the central nervous system, such as appetite control, stress response, anxiety, or depression. Thus, determining the specific role of the CRF_2 receptor within the brain not only will contribute to our understanding of central CRF systems but also may lead to the development of CRF receptor subtype-specific drugs.

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