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^{125}I-Tyr^{0}-Sauvagine: A Novel High Affinity Radioligand for the Pharmacological and Biochemical Study of Human Corticotropin-Releasing Factor$_{2\alpha}$ Receptors

DIMITRI E. GRIGORIADIS, XIN-JUN LIU, JOAN VAUGHN, SCOTT F. PALMER, C. DIANE TRUE, WYLIE W. VALE, NICHOLAS LING, and ERROL B. DE SOUZA

Neurocrine Biosciences, Inc., San Diego, California 92121 (D.E.G., X.-J.L., N.L., E.B.DeS.), The Clayton Foundation for Peptide Biology, Salk Institute, La Jolla, California 92037 (J.V., W.W.V.), and DuPont-New England Nuclear Products, North Billerica, Massachusetts 01862 (S.F.P., C.D.T.)

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SUMMARY

Corticotropin-releasing factor (CRF) receptors encoded by two distinct genes have recently been identified and termed CRF_1 and CRF_2. CRF and the non-mammalian-related peptide sauvagine bind to and activate CRF_1 receptors with high affinity and equal potency. Although CRF is significantly weaker at the CRF_2 receptor, sauvagine retains its high affinity interactions with this receptor subtype. We expressed the human CRF_1 and CRF_2 receptor subtypes in stable cell lines and characterized ^{125}I-Tyr^{0}-sauvagine, a high affinity radiolabel suitable for the pharmacological and functional profiles of these proteins. ^{125}I-Tyr^{0}-sauvagine has high affinity (200–400 pM) for CRF_1 receptors and demonstrates a pharmacological profile identical to that of ^{125}I-Tyr^{0}-ovine CRF-labeled CRF_1 receptors. ^{125}I-Tyr^{0}-sauvagine binding to human $CRF_{2\alpha}$ receptors is saturable and of high affinity (K_D = 100–300 pM) and demonstrates guanine nucleotide sensitivity typical of agonist binding to receptors. The pharmacological profile of ^{125}I-Tyr^{0}-sauvagine binding to $CRF_{2\alpha}$ receptors with respect to inhibition by CRF-related analogs is similar to the agonist profile of potencies obtained by measurements of cAMP production stimulated by these analogs in $CRF_{2\alpha}$ expressing cell lines and distinct from the profile of the CRF_1 receptor subtype. Thus, the related nonmammalian peptides sauvagine and urotensin have high affinity and rat/human CRF and ovine CRF have lower affinity for CRF_2 receptors labeled with ^{125}I-Tyr^{0}-sauvagine. Because the distribution of CRF_1 and $CRF_{2\alpha}$ receptors has been demonstrated to be distinct, suggesting selective functional roles for each receptor subtype, the ability to label $CRF_{2\alpha}$ receptors with ^{125}I-Tyr^{0}-sauvagine *in vitro* represents a unique opportunity for the discovery of subtype-selective nonpeptide ligands, which would presumably target different aspects of CRF-mediated disorders. We have thus identified and characterized a novel high affinity radioligand for the labeling of CRF_2 receptors.

CRF produced and secreted primarily from parvocellular neurons of the paraventricular hypothalamic nuclei is the primary regulator of the release of adrenocorticotropic hormone and other proopiomelanocortin derivatives from the anterior pituitary gland. In addition to its endocrine effects, immunohistochemical localization of CRF has demonstrated that the hormone has a broad extrahypothalamic distribution in the central nervous system (1–4). CRF produces a wide array of autonomic, electrophysiological, and behavioral effects and fulfills all of the criteria for a *bona fide* neurotransmitter (for a review, see Refs. 5–8). The cloning of the CRF_1 receptor and, more recently, two splice forms of a second member of the CRF receptor family, designated $CRF_{2\alpha}$ and $CRF_{2\beta}$, has allowed the discrete localization of the mRNA of these receptor subtypes and demonstrated a clear and distinct pattern of distribution in rat brain and periphery. Further studies have also suggested that in addition to its neuromodulatory and neuroendocrine actions, CRF may play a significant role in a variety of peripheral functions, including cardiovascular function, inflammation, reproduction, and integration of the response of the immune system to stressors (8–12).

For more than a decade, receptors for CRF have been studied using radioligand binding and classic biochemical techniques in an attempt to elucidate their structure-function relationship and define their potential as sites of thera-

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ABBREVIATIONS: CRF, corticotropin-releasing factor; oCRF, ovine corticotropin-releasing factor; hCRF, human corticotropin-releasing factor; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; GTP-γ-S, guanosine-5′-*O*-(3-thio)triphosphate; D-Phe-CRF, D-Phe^{12},Nle^{21,38},Ala^{32}-human corticotropin-releasing factor(12–41); CHO, Chinese hamster ovary; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

peutic intervention for a variety of indications. Although these studies yielded a great deal of information, they could not conclusively account for the myriad functions of CRF described in both the central nervous system and the periphery. Several groups have simultaneously reported the cloning of the CRF_1 receptor from rat, mouse, and human (13–16). The cDNA encoded a protein of 415 amino acids and the mRNA distribution for this receptor subtype correlated well with the known distribution of CRF binding sites determined by autoradiographic localization in that expression was highest in the pituitary, cerebral cortex, and cerebellum. Indeed, when this receptor was expressed in cells, it exhibited an identical pharmacological profile to that previously described in brain and pituitary.

A second CRF receptor family member was subsequently reported (CRF_2) from both rat (17) and mouse (18, 19) that demonstrated an overall 71% homology with the cloned CRF_1 receptor and ~30% homology with other members of this neuropeptide receptor superfamily (17). Interestingly, this receptor subtype was found to exist in two alternatively spliced forms, the $CRF_{2\alpha}$, a 411-amino acid protein, and the $CRF_{2\beta}$, a 431-amino acid protein. These two splice variants differ only in their 5′ sequence in that the first 34 amino acids of the $CRF_{2\alpha}$ receptor are replaced with an open reading frame encoding 54 different amino acids. Both of these forms were reported in rat (17); however, only the $CRF_{2\beta}$ form has been described in mouse (18, 19), and only the $CRF_{2\alpha}$ form has been found in humans (20). We recently demonstrated that the anatomic distribution of these CRF_2 receptors is completely distinct from that of the CRF_1 receptors (21). Thus, although the CRF_1 receptor is widely distributed within the central nervous system, including cortical, cerebellar, and sensory relay structures, the distribution of the CRF_2 mRNA demonstrates a more discrete localization to specific subcortical structures within limbic areas of the brain, including lateral septum and various hypothalamic nuclei. Preliminary studies have further demonstrated that $CRF_{2\alpha}$ receptors display a unique pharmacological profile when expressed in cells, which serves to further separate and distinguish them from the CRF_1 receptor subtype (22). These differences in the anatomic and pharmacological profile, coupled with the lack of molecules available as tools with high affinity for the CRF_2 receptor subtype, are primarily responsible for the inability to clearly elucidate the physiological nature of this family of receptors.

A key component in the elucidation of receptor pharmacology, structure, and function is the availability of specific molecules that can be used as tools in the characterization of these proteins. To date, the only radiolabels commercially available for the study of the CRF system *in vitro* have been the rat/human or ovine forms of CRF itself. ^{125}I-oCRF binds with high affinity to the CRF_1 receptor and, as a result of its lower affinity for the CRF_2 receptor and the CRF binding protein (22, 23), has been used successfully for the selective study of the CRF_1 receptor. ^{125}I-r/hCRF has high affinity for both the CRF_1 receptor (24) and the CRF binding protein (23) but lower affinity for the CRF_2 receptor (22), limiting its use for the characterization of this subtype. The non-mammalian CRF-related peptide sauvagine has been demonstrated to have low affinity for the CRF binding protein (~10-20 nM; Ref. 23) and equally high affinity for both CRF receptor subtypes (17), establishing its potential as a CRF_2 receptor ligand. We now report the characterization of a novel labeled ligand (^{125}I-Tyr^0-sauvagine) that has high affinity for both the CRF_1 and $CRF_{2\alpha}$ receptor subtypes and low affinity for the CRF binding protein, allowing for the first time the labeling of the $CRF_{2\alpha}$ receptor with high affinity *in vitro* and providing a pharmacological tool for the study of this unique and novel receptor subtype.

Materials and Methods

All chemicals and standard reagents were purchased from either Sigma Chemical (St. Louis, MO) or Fisher (Los Angeles, CA) unless specifically otherwise stated. All unlabeled peptides were synthesized in-house.

Synthesis and radioiodination of Tyr^0-sauvagine. Sauvagine was synthesized in-house *de novo*, including the addition of a tyrosine residue at the amino terminus (0 position) to facilitate radioiodination. The radioiodination of the resulting Tyr^0-sauvagine was performed at DuPont-New England Nuclear (Boston, MA]). Tyr^0-Sauvagine was radiolabeled using standard lactoperoxidase methods. Briefly, Tyr^0-sauvagine was reacted with Na^{125}I (catalog No. NEZ-033H) in the presence of lactoperoxidase and hydrogen peroxide. The products were purified by reverse-phase high performance liquid chromatography using an acetonitrile/trifluoroacetic acid gradient. ^{125}I-Tyr^0-Sauvagine was then collected and diluted to a concentration of 50 μCi/ml on assay in water/acetonitrile (65:35) containing 0.2% trifluoroacetic acid, 0.2% bovine serum albumin, and 0.05 M β-mercaptoethanol. The product (NEX-306) was stored at 4° until use. The radioiodination typically yielded a specific activity of 2200 Ci/mmol for the final product with a purity of >95%; preliminary studies have determined that the labeled compound is stable for >6 weeks.

Stable expression and generation of $CRF_{2\alpha}$ receptors in CHO cells. The full-length human $CRF_{2\alpha}$ receptor cDNA was subcloned into the mammalian expression vector pCDM-7amp under the control of an RSV promoter and transfected into CHO cells using lipofectamine as described previously (20). The highest expressing clones were identified by a whole-cell sauvagine-stimulated cAMP assay (22) and expanded to T-150 cell culture flasks. The $CRF_{2\alpha}$ receptor expression level was further amplified by cotransfection with dihydrofolate reductase through a series of selective pressures in the presence of dihydrofolate reductase. Transfected cells were grown in T-150 tissue culture flasks to 90% confluency, trypsinized (0.5% trypsin), and transferred into roller bottles for bulk cell production. Cells were incubated for 7 days at 37° in roller bottles (final volume, 300 ml); 24 hr before harvest, the cells were treated with sodium butyrate (Sigma) to further amplify the protein expression (25). On the day of harvest, roller bottles were rinsed once with 300 ml of PBS to remove detached nonviable cells, and the remaining adherent cells were detached from the roller bottles using 5 mM EDTA and placed into 50-ml conical centrifuge tubes. Cells were washed once in PBS, and the viable cells were counted using Trypan Blue exclusion. Whole cells were finally placed into Eppendorf tubes (50 million cells/tube), and the pellets were frozen at −80° until use.

Membrane preparation. On the day of assay, pellets of whole cells (as described above) were thawed on ice and diluted in 5–7 ml of ice-cold PBS containing 10 mM $MgCl_2$ and 2 mM EGTA, pH 7.0, at 22° (tissue buffer) and homogenized at 25,000 rpm for 15 sec on ice using a Polytron (Brinkmann, Westbury, NY) tissue homogenizer. Membrane homogenates were then washed twice by centrifugation (30,000 × *g* for 10 min at 4°) in the same buffer, and the final pellets were resuspended in buffer to a working concentration of ~3 million cells/ml or ~1.2 mg/ml of protein. Protein concentrations were determined according to the method of Bradford (26) using the bicinchoninic acid protein determination assay from Pierce Chemical (Rockford, IL) and bovine serum albumin as a standard. Typically,

2.5×10^6 CHO cells yielded 1 mg of protein with the final protein concentration in the assay being 50 μg/tube.

Stimulation of cAMP production by sauvagine. Adenylate cyclase activity was performed using cells transfected with either the human CRF_1 or $CRF_{2\alpha}$ receptor. Cells were plated onto 24-well cell culture plates and grown to confluency. On the day of assay, the plates were removed from the incubator, the medium was aspirated, and the cells were washed once with PBS. Assays were carried out at 37° for 1 hr in a final volume of 0.5 ml in assay buffer containing Dulbecco's modified Eagle's medium, 2 mM L-glutamine, 20 mM HEPES, and 1 mM 3-isobutyl-1-methylxanthine. In stimulation studies, various concentrations of CRF-related and -unrelated peptides were incubated with the cells to establish the pharmacological rank-order profile of this receptor subtype. For the functional assessment of antagonists [α-helical CRF(9–41) or D-Phe-CRF(12–41)], 10 nM sauvagine (causing ~60-80% stimulation of cAMP production) was incubated along with various concentrations of competing peptides (10^{-12} to 10^{-6} M). After the incubations, the medium was aspirated, the wells were rinsed once gently with fresh medium and aspirated. Intracellular cAMP was extracted from the cells by the addition of 300 μl of a solution of 95% ethanol containing 20 mM HCl and incubated at −20° for 16–18 hr. The solution was then transferred to 1.5-ml Eppendorf tubes, and the wells were washed with an additional 200 μl of 95% ethanol containing 20 mM HCl and pooled with the first fraction. The samples were lyophilized and resuspended with 500 μl of sodium acetate buffer. The measurement of cAMP in the samples was performed using a single antibody cAMP radioimmunoassay kit (Biomedical Technologies, Stoughton, MA).

^{125}I-Tyr^0-sauvagine binding studies. Radioligand binding of ^{125}I-Tyr^0-sauvagine in transfected CHO cells was performed essentially as previously described in detail for the binding of either ^{125}I-r/hCRF or ^{125}I-oCRF to many different tissues (for a review, see Refs. 24 and 27). All drugs and reagents (i.e., guanine nucleotides) were made up in assay buffer, which was the same as the tissue buffer described above with the addition of 0.15 mM bacitracin, pH 7.0, at 22°. Briefly, 1.5-ml Eppendorf tubes received (in order) 100 μl of buffer (with or without competing peptides, guanine nucleotides, and so on), 50 μl of ^{125}I-Tyr^0-sauvagine, and 150 μl of the membrane suspension as described above, for a total assay volume of 300 μl. The assay was incubated at equilibrium routinely for 2 hr at 22° as determined by direct kinetic experiments described below. Nonspecific binding was determined in the presence of 1 μM unlabeled peptide antagonist D-Phe-CRF. Reactions were terminated by centrifugation in a Beckman microfuge for 10 min at 12,000 × *g*. The resulting pellets were washed gently with 1 ml of ice-cold PBS containing 0.01% Triton X-100 and centrifuged again for 10 min at 12,000 × *g*. The supernatants were aspirated, and the tubes were cut just above the pellet, placed into 12- × 75-mm polystyrene tubes, and monitored for radioactivity in a Packard Cobra II γ-counter at ~80% efficiency.

Kinetic studies were performed to determine the association and dissociation rate constants for ^{125}I-Tyr^0-sauvagine and determine the optimal time for equilibrium binding. Association studies were initiated with the addition of the membrane suspension to triplicate tubes containing the radiolabel (100–200 pM final concentration) and buffer, with or without 1 μM D-Phe-CRF to define nonspecific binding. Tubes were allowed to incubate for various times before centrifugation and washing of the membranes. Specific binding was calculated for each time point, and the rate constant for the association was determined. For dissociation experiments, tubes were set up as described for the association assays and allowed to incubate for 2 hr at 22°. After the 2-hr incubation, 1 μM D-Phe-CRF (final concentration) was added to all tubes (total and nonspecific) to initiate dissociation of the label in a final volume of 10 μl to minimize the effects of dilution. The tubes again were centrifuged at various times, and the specific binding was used to calculate the dissociation rate constant as described below.

For competition studies, tubes received (in order) 50 μl of buffer, 50 μl of competing peptides (final concentration, 1 pM to 1 μM), 50 μl of ^{125}I-Tyr^0-sauvagine (final concentration, 100–200 pM), and 150 μl of membrane suspension as described above. Homogenates were typically allowed to incubate for 2 hr at 22°, and the reaction was terminated by separation of the bound from free radioligand by centrifugation as described above.

For Scatchard saturation studies, tubes received (in order) 50 μl of buffer, 50 μl of ^{125}I-Tyr^0-sauvagine (final concentration, 10 pM to 2 nM), and 50 μl of buffer with or without D-Phe-CRF to define nonspecific binding. All assays were carried out at equilibrium at 22° as determined by the association experiments described above. Specific binding was determined at each concentration of radioligand in the presence of 1 μM D-Phe-CRF(12–41). This concentration was chosen from direct competition studies demonstrating that at a concentration of 1 μM, D-Phe-CRF could displace >95% of ^{125}I-Tyr^0-sauvagine binding from CHO cells expressing the $CRF_{2\alpha}$ receptor.

Data analysis. All data analyses, including Scatchard, competition and kinetic experiments, were analyzed using the iterative nonlinear least-squares curve-fitting program Prism (GraphPAD, San Diego, CA). The saturation analyses of ^{125}I-Tyr^0-sauvagine binding yielded K_d values that were subsequently used in the calculation of the apparent K_i values for competing ligands in competition assays performed under identical conditions. For competition curves, the data were routinely fit to single- and multiple-site models, and the fits were compared statistically to determine whether a more-complex data model was justified.

Results

Pharmacological profile of cAMP production in cells expressing human CRF_1 and $CRF_{2\alpha}$ receptors. Cells were stably transfected with either human CRF_1 or $CRF_{2\alpha}$ receptors and used to determine the pharmacological profiles of the two receptor subtypes. Because both receptors have been previously demonstrated to stimulate cAMP production, CRF-related and -unrelated peptides were used to define the rank order of potencies for these subtypes. The peptides r/hCRF and oCRF had approximately equal potencies in stimulating cAMP formation in cells transfected with the CRF_1 receptor with EC_{50} values of 3.5 ± 1.3 and 9.7 ± 1.2 nM, respectively (Table 1). In addition, the nonmammalian analogs of CRF, sauvagine (frog) and urotensin I (sucker fish), also exhibited similar potencies in stimulating the production of cAMP with EC_{50} values of 3.4 ± 1.5 and 6.3 ± 1.6 nM, respectively (Table 1). In contrast, although the nonmammalian forms of CRF (sauvagine and urotensin I) retained their potencies in stimulating cAMP production in cells transfected with the human $CRF_{2\alpha}$ receptor (EC_{50} values = 1.5 ± 0.8 and 1.4 ± 1.2 nM, respectively), r/hCRF and oCRF were significantly less potent in their ability to stimulate cAMP formation, exhibiting EC_{50} values of 13.2 ± 4.6 and 61.9 ± 15.8 nM, respectively (Table 1). The deamidated form of CRF [r/hCRF(1–41)-OH], which has been well documented to be inactive at the CRF_1 receptor, was again devoid of all activity at the $CRF_{2\alpha}$ receptor, as were ligands specific for the CRF binding protein [r/h(6–33) and r/hCRF(9–33)]) or the unrelated peptides such as growth hormone-releasing factor, vasoactive intestinal peptide, and arginine vasopressin (Table 1).

Affinity of Tyr^0-sauvagine for CRF_1 receptors labeled with ^{125}I-oCRF. To determine the binding characteristics of ^{125}I-Tyr^0-sauvagine, both the native form, sauvagine, and the tyrosine-extended form to be used for subsequent labeling, Tyr^0-sauvagine, competed for ^{125}I-oCRF binding to

TABLE 1

Rank order of potencies of CRF-related and -unrelated peptides for stimulation of cAMP production in cells transfected with human CRF_1 and $CRF_{2\alpha}$ receptors and inhibition of [^{125}I]sauvagine binding in cells transfected with human $CRF_{2\alpha}$ receptors

Stimulation of cAMP production in cells transfected with either the human CRF_1 or $CRF_{2\alpha}$ receptor was compared with the direct inhibition of [^{125}I]sauvagine binding in $CRF_{2\alpha}$-expressing cells. For both the CRF_1 and $CRF_{2\alpha}$ receptors, production of cAMP was performed as described in Materials and Methods. Values represent the mean ± standard error for three independent experiments performed in duplicate.

Peptide	Stimulation of cAMP production (EC_{50})		Inhibition of [^{125}I]sauvagine binding
	$hCRF_1$ cells	$hCRF_{2\alpha}$ cells	$CRF_{2\alpha}$ cells (K_i)
		nM	
Urotensin I	6.3 ± 1.6	1.5 ± 0.8	2.2 ± 0.1
Sauvagine	3.4 ± 1.5	1.4 ± 1.2	4.3 ± 0.9
Tyr^0-Sauvagine	ND	ND	5.8 ± 0.8
D-Phe hCRF(12-41)	NA	NA	24.5 ± 1.9
α-hel oCRF(9-41)	NA	NA	96.2 ± 22.1
r/hCRF	3.5 ± 1.3	13.2 ± 4.6	30.7 ± 9.7
oCRF	9.7 ± 1.2	61.9 ± 15.8	184.6 ± 26.5
Tyr^0-oCRF	ND	ND	305.4 ± 30.8
r/hCRF(6-33)	>10,000	>10,000	>10,000
oCRF(9-33)	>10,000	>10,000	>10,000
r/hCRF(1-41)OH	>10,000	>10,000	>10,000
Growth hormone-releasing factor	>10,000	>10,000	>10,000
Vasoactive intestinal peptide	>10,000	>10,000	>10,000
Arginine vasopressin	>10,000	>10,000	>10,000

NA, not active in stimulating cAMP production; ND, not determined.

cells transfected with the human CRF_1 receptor. As shown in Fig. 1, the binding of ^{125}I-oCRF to membranes from cells transfected with the CRF_1 receptor could be competed by both sauvagine and Tyr^0-sauvagine with high and approximately equal affinities (K_i = ~5 nM). For comparison, the inhibition of ^{125}I-oCRF by the putative peptide antagonist D-Phe-CRF(12–41) is also shown and had an affinity of ~25 nM (See Fig. 1 and Table 1). All competition curves were monophasic, suggesting that the inhibition was to a single homogeneous class of binding site (Fig. 1). The inhibition values at the highest doses tested for all three compounds (1 μM) were similar, suggesting that the specific binding for these compounds to the expressed CRF_1 receptors were identical. In addition, functional studies using both cAMP stimulation studies in transfected cells and adrenocorticotropic hormone release studies from cultured rat pituitary cells demonstrated that Tyr^0-sauvagine had agonist properties similar to that of the native peptide (data not shown). Furthermore, the K_i values determined in the cells expressing the CRF_1 receptor are consistent and in agreement with the plethora of information available for these compounds in the brain and periphery of various species examined.

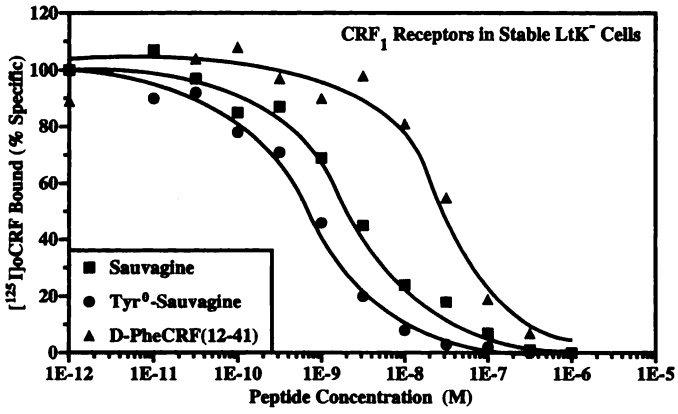

Fig. 1. Competition of Tyr^0-sauvagine for ^{125}I-oCRF binding in cells stably transfected with human CRF_1 receptors. Tyr^0-sauvagine was competed for ^{125}I-oCRF binding in cells transfected with the human CRF_1 receptor to determine whether the tyrosine extension would affect the binding profile. Tyr^0-sauvagine had approximately the same affinity as the native form of sauvagine (K_i = 0.8–1 nM). The putative peptide receptor antagonist D-PheCRF(12–41) is also shown as a reference. All peptides inhibited the binding of ^{125}I-oCRF to the same base-line value, indicating that the inhibition characteristics were the same for all three peptides. Data represent mean values of duplicate determinations, and the experiment was repeated three times with identical results.

Kinetic analysis of ^{125}I-Tyr^0-sauvagine binding. ^{125}I-Sauvagine bound rapidly and reversibly to CHO cells stably expressing the human $CRF_{2\alpha}$ receptor. Association experiments revealed that ^{125}I-Tyr^0-sauvagine (50–100 pM) binding reached steady state equilibrium within 60–120 minutes at 22°, with the binding remaining at equilibrium for ≤4 hr. The association rate constant (K_{+1}) was determined (assuming pseudo-first-order kinetics) to be 0.415 min^{-1} $nmol^{-1}$ (Fig. 2). The dissociation rate constant of ^{125}I-Tyr^0-sauvagine (50–100 pM) binding was estimated after a 120-min incubation at 22° [after equilibrium binding was achieved] by the addition of 1 μM D-Phe-CRF(12–41)] to be 0.0252 min^{-1} (Fig. 2, *inset*). The affinity of ^{125}I-Tyr^0-sauvagine (K_d) for the $CRF_{2\alpha}$ receptor was then calculated from these kinetic experiments ($K_d = K_{-1}/K_{+1}$) to be 60.6 ± 0.01 pM and was in agreement with the affinity K_d value obtained from direct saturation experiments (see below). In all subsequent experiments, incubation times for the binding of ^{125}I-Tyr^0-sauvagine to $CRF_{2\alpha}$ receptors were routinely set at 120 min at 22°.

Saturation of ^{125}I-Tyr^0-sauvagine binding. The saturable binding of ^{125}I-Tyr^0-sauvagine to cloned and stably expressed $CRF_{2\alpha}$ receptors is shown in Fig. 3. ^{125}I-Sauvagine binding was both specific and saturable and displayed a mean dissociation constant (K_d) of 162 ± 40 pM. Saturation analysis demonstrated that the binding of ^{125}I-Tyr^0-sauvagine was consistent with binding to a homogeneous population of binding sites, with an average number of receptors bound (Bmax) equal to 178 ± 55 fmol/mg protein. The binding was also highly specific in the expressed cell line with the specific

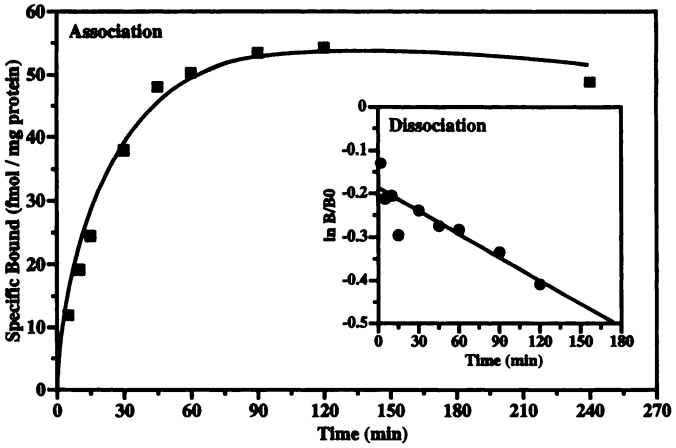

Fig. 2. Association and dissociation of ^{125}I-Tyr^0-sauvagine binding to human $CRF_{2\alpha}$ receptors expressed in stable CHO cell lines. For association experiments, cell membrane homogenates were incubated at 22° with ~100-200 pM ^{125}I-Tyr^0-sauvagine for various times (as described in Materials and Methods). Nonspecific binding was defined in the presence of 1 μM D-Phe-CRF(12–41) at each time point. The association rate constant (K_{+1}) for ^{125}I-Tyr^0-sauvagine was determined (assuming pseudo-first-order kinetics) by plotting [ln $Be/(Be - B)$] versus time, where Be is the amount specifically bound (fmol/mg of protein) at equilibrium, and B is the amount specifically bound at any given time point. The association rate constant was calculated from the following equation: [$K_{ob} = K_{-1} = K_{+1}\cdot(C_L)$], where K_{ob} is the slope of the association, K_{-1} is the dissociation rate constant, and C_L is the concentration of ligand used. *Inset*, dissociation of ^{125}I-Tyr^0-sauvagine. After equilibrium, dissociation of ^{125}I-Tyr^0-sauvagine was initiated by the addition of 1 μM D-Phe-CRF(12–41), and the reaction was stopped at various times by centrifugation. The specific binding was calculated and the dissociation constant was determined from the following equation: [ln $B/B_0 = K_{-1}\cdot t$], where B is the amount specifically bound at any given time point, B_0 is the amount specifically bound at equilibrium, and t is time. This experiment was repeated once with identical results.

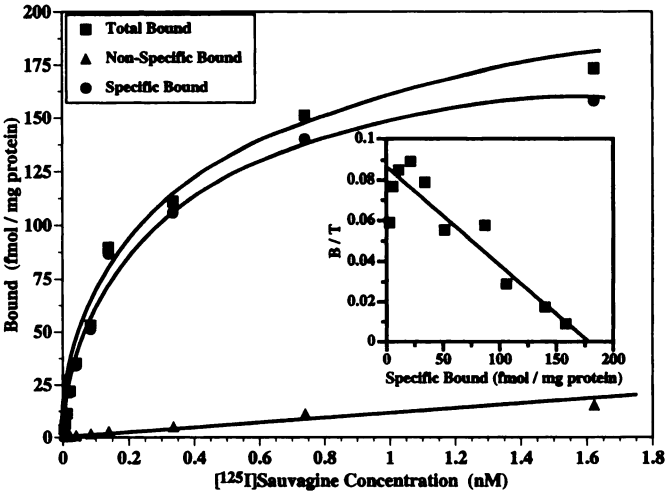

Fig. 3. Saturation and Scatchard analyses of ^{125}I-Tyr^0-sauvagine binding to human $CRF_{2\alpha}$ receptors expressed in stable CHO cell lines. Human $CRF_{2\alpha}$ receptor-transfected CHO cell membranes were incubated with 10–12 concentrations of ^{125}I-Tyr^0-sauvagine (1 pM–2 nM) at 22° as described in Materials and Methods. Nonspecific binding was defined in the presence of 1 μM D-Phe-CRF(12–41) at each concentration. K_D and B_{max} values were estimated by the nonlinear regression analysis package Prism (GraphPAD) *Points*, mean of duplicate determinations. Graph is representative of four independent experiments that yielded similar results.

signal [defined in the presence of 1 μM D-Phe-CRF(12–41)] at saturation being >80% and at lower concentrations (those approximating the K_d value) being >95% (Fig. 3).

Pharmacological characterization of ^{125}I-Tyr^0-sauvagine binding to human expressed $CRF_{2\alpha}$ receptors. To demonstrate whether the pharmacological profile of ^{125}I-Tyr^0-sauvagine binding to $CRF_{2\alpha}$ receptors was the same as the rank order profile determined in the cAMP production functional assays describe above, ^{125}I-Tyr^0-sauvagine binding was competed by a variety of CRF-related and -unrelated peptides in homogenates of $CRF_{2\alpha}$-transfected cells. Fig. 4 demonstrates the rank order of potencies for CRF-related peptides: urotensin I ≈ sauvagine ≈ Tyr^0-sauvagine > D-Phe-CRF(12–41) ≈ r/hCRF > α-helical oCRF(9–41) > oCRF > Tyr^0-oCRF. This rank order of potencies was similar to the rank order profile obtained in the *in vitro* functional tests measuring the stimulation of cAMP production in these same human $CRF_{2\alpha}$ receptor-expressing cell lines (Table 1). Consistent with the *in vitro* functional cAMP assays and the previously reported profile, all of the fragments of CRF peptides and unrelated peptides were without activity, again confirming the unique pharmacological profile of this receptor subtype (Table 1).

Guanine nucleotide sensitivity of ^{125}I-Tyr^0-sauvagine binding. Sauvagine has been demonstrated to be an agonist at both the human CRF_1 and $CRF_{2\alpha}$ receptors, primarily due to its ability to stimulate cAMP production through a specific interaction with the expressed receptors (Table 1). Because these receptors seem to be G protein linked, guanine nucleotides should be able to reduce the specific binding of ^{125}I-Tyr^0-sauvagine at the level of the receptor. GTP and its nonhydrolyzable analogs, Gpp(NH)p and GTP-γ-S, were incubated in increasing concentrations to determine the guanine nucleotide sensitivity of ^{125}I-Tyr^0-sauvagine binding to human $CRF_{2\alpha}$ receptors in expressed cell lines. As shown in Fig. 5, all of the guanine nucleotides could inhibit the binding of ^{125}I-Tyr^0-sauvagine to the same degree. This inhibition was ~60% relative to the total specific signal obtained using the putative CRF receptor antagonist D-Phe-CRF(12–41). Furthermore, the rank order of potencies for the guanine nucleotides was typical for G protein-coupled receptors in that the nonhydrolyzable forms of GTP were more potent inhibitors of the binding. The adenosine analog ATP also could inhibit the binding but only at high concentrations (EC_{50} >100 μM). Thus, the rank order of potencies observed was GTP-γ-S > Gpp(NH)p ≥ GTP ≫ ATP, with apparent EC_{50} values of ~50, ~160, and ~200 nM, respectively (Fig. 5).

Discussion

In this report, we demonstrated the use of the novel radioligand ^{125}I-Tyr^0-sauvagine in the study of the recently cloned human $CRF_{2\alpha}$ receptor. This receptor is part of the CRF family within the superfamily of neuropeptide receptors, including those for growth hormone-releasing hormone, glucagon, secretin, vasoactive intestinal peptide, parathyroid hormone, pituitary adenylate cyclase activating peptide, and calcitonin. In addition, the protein was characteristic of other G protein-linked membrane-bound receptors, with seven putative transmembrane domains and a short third intracytoplasmic loop, suggesting that this protein is coupled to a stimulatory G protein. Although exhibiting >70% homology with the CRF_1 receptor, the pharmacological profile deter-

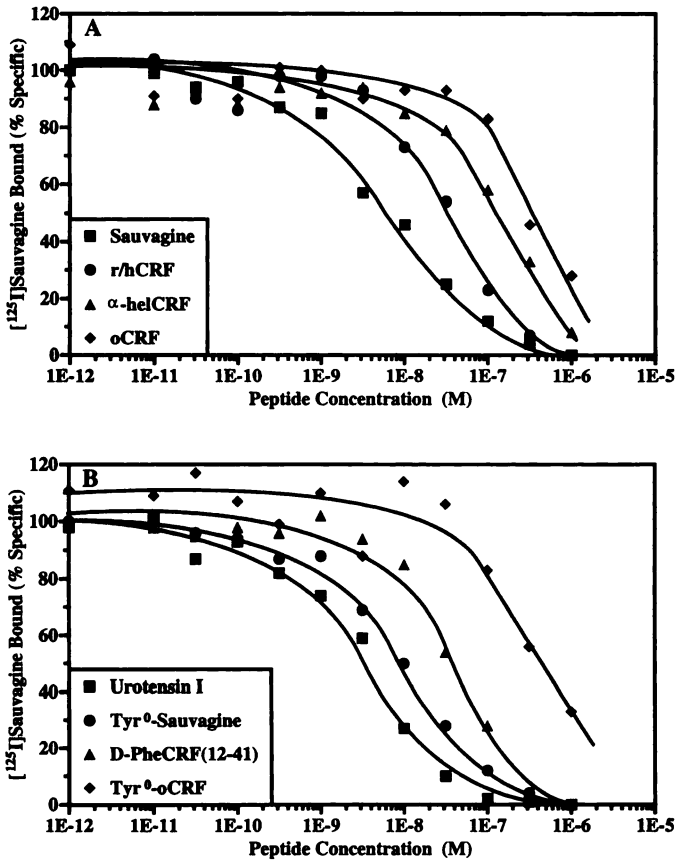

Fig. 4. Competition of CRF-related peptides for ^{125}I-Tyr^0-sauvagine binding to human $CRF_{2\alpha}$ receptors expressed in stable CHO cell lines. Human $CRF_{2\alpha}$ receptor-transfected CHO cell membranes were incubated with ^{125}I-Tyr^0-sauvagine (200 pM) at 22° with varying concentrations of competing peptides and analyzed for their ability to inhibit the binding. The tyrosinated forms of sauvagine and oCRF were used to determine the effects of extending the peptides by a tyrosine residue at the amino terminus and found to be equipotent with the native forms. *Points*, mean of duplicate experiments. The experiments were replicated three times with an identical rank order.

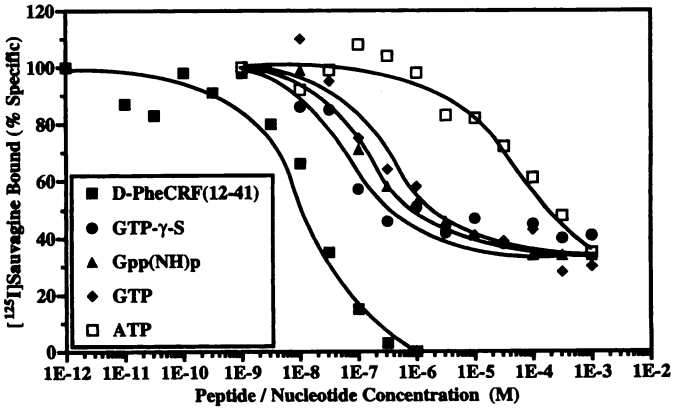

Fig. 5. Effects of guanine nucleotides on the binding of ^{125}I-Tyr^0-sauvagine to human $CRF_{2\alpha}$ receptors expressed in stable CHO cell lines. GTP and its nonhydrolyzable analogs Gpp(NH)p and GTP-γ-S were examined for their ability to inhibit the binding of ^{125}I-Tyr^0-sauvagine to the $CRF_{2\alpha}$ receptor. All guanine nucleotides were able to inhibit the binding to the same extent at ~60% of the total specific signal as defined by D-Phe-CRF(12–41). The inhibition of binding by D-Phe-CRF(12–41) is shown for reference. The adenosine nucleotide ATP could also compete for the binding but was ~2 orders of magnitude lower in potency than GTP. The data are the mean of duplicate determinations and representative of three independent experiments with similar results.

mined for this receptor subtype was quite different in that oCRF and r/hCRF were relatively weak in their abilities to stimulate cAMP production in transfected cell lines (17, 20). In addition to the structural and pharmacological differences between the CRF_1 and $CRF_{2\alpha}$ receptors, this receptor subtype had a localization in the central nervous system that was quite distinct from the known and well characterized distribution of the CRF_1 receptor (21, 28). Thus, this receptor not only is derived from a different gene product but also exhibits a unique distribution within the central nervous system as well as a distinct pharmacological profile from the CRF_1 receptor, providing both a pharmacological and an anatomic basis for the multiple functions of CRF as a hormone and a neurotransmitter.

To date, there have been numerous reports that have described the radioligand binding characteristics of CRF receptors using the available radioligands in a variety of tissues (for reviews, see Refs. 5, 8, 24, and 29). However, before the recent elucidation of the CRF_2 subfamily of receptors, all of the *in vitro* and *in vivo* characteristics were ascribed to a single receptor subtype. Fortunately, this body of data is unaffected by the discovery of a second family member because the ligands that were used seem to have selectivity for the CRF_1 receptor subtype. However, the commercially available radioligands for CRF receptors had relatively low affinity for the $CRF_{2\alpha}$ receptor subtype and were not useful in identifying and characterizing this subtype. Based on the *in vitro* functional studies, sauvagine seemed to be a viable candidate for radiolabeling. However, because the native form of sauvagine (a 40-amino acid peptide) contained neither a histidine residue nor a tyrosine residue suitable for radioiodination, we extended the amino acid sequence at the amino terminus by the *de novo* synthesis of the peptide and the subsequent addition of a tyrosine residue at position 0. Before radioiodination, the unlabeled tyrosinated peptide (Tyr^0-sauvagine) was assessed and found to be equipotent with the native form in its ability to either bind to the CRF_1 receptor or stimulate cAMP in cells transfected with the $CRF_{2\alpha}$ receptor. Thus, the extension of this peptide by one amino acid at the amino terminus did not alter its ability to interact with the CRF receptor, and ^{125}I-Tyr^0-sauvagine was synthesized and radioiodinated to a high specific activity.

^{125}I-Tyr^0-Sauvagine was found to bind reversibly, saturably, and with high affinity to both the human CRF_1 and $CRF_{2\alpha}$ receptor subtypes expressed in mammalian cell lines. In addition, the specific signal for the labeling of the human $CRF_{2\alpha}$ receptors was >85% over the entire concentration range of the radioligand, suggesting very low nonspecific binding. Kinetic analyses for association of the label confirmed that the radioligand bound in a reversible and time-dependent manner, reaching equilibrium within 60 min, with the binding being stable for ≥4 hr at 22°. After a 2-hr incubation to equilibrium, the putative CRF receptor antagonist D-Phe-CRF(12–41) could effectively dissociate bound ^{125}I-Tyr^0-sauvagine from the $CRF_{2\alpha}$ receptor, with a half-life of ~30 min. This clearly demonstrated that the binding of ^{125}I-Tyr^0-sauvagine to human $CRF_{2\alpha}$ receptors was of a reversible nature and could be competitively displaced once equilibrium had been achieved.

CRF-related and unrelated peptides were used to compete

for the binding of ^{125}I-Tyr^0-sauvagine in cells transfected with the human $CRF_{2\alpha}$ receptor. The pharmacological rank order binding profile of these peptides was essentially identical to the *in vitro* effects of the same unlabeled peptides in the production of cAMP in cells expressing the receptor. That is, the nonmammalian analogs sauvagine and urotensin I, which were more potent in stimulation of cAMP production, were also more potent at inhibiting the binding of ^{125}I-Tyr^0-sauvagine than oCRF or r/hCRF. The $CRF_{2\alpha}$ receptor, however, retained its specificity for receptor ligands in that peptides that have been shown to bind to the CRF binding protein [r/hCRF(6–33 or oCRF(9–33)] or the deamidated form of CRF, r/hCRF(1–41)OH, were completely unable to inhibit the binding. Furthermore, peptides related to the superfamily of these receptors, such as growth hormone-releasing factor, vasoactive intestinal peptide, and arginine vasopressin, were also completely ineffective in inhibiting ^{125}I-Tyr^0-sauvagine binding. These data clearly suggest that although there is a distinct pharmacological difference between the two receptor subtypes of the same family (in terms of their rank order profile), they still must share some structural similarities. Further study will be required to determine the precise common structural features of these two family members. In addition to the nonmammalian CRF-like peptides having equal affinity for CRF_1 and $CRF_{2\alpha}$ receptors, the putative antagonists for CRF receptors, D-Phe-CRF(12–41) and α-helical CRF(9–41), exhibited approximately equal affinity for the two receptor subtypes (Table 1). This not only further serves to identify these two subtypes as close family members but also offers the opportunity for the design of molecules that can interact specifically with one of the receptors or both.

In addition to the similarities between the two receptor proteins and the peptide affinities in the binding and cAMP profiles described above, the human $CRF_{2\alpha}$ receptor conforms to the superfamily of G protein-coupled receptors. With a typical short third intracytoplasmic loop, this protein was predicted to function through a stimulatory G protein. G proteins are known to play an integral role in the regulation of receptor-mediated events for many receptor systems (for a review, see Ref. 30). Two prominent actions of guanine nucleotides related to neurohormone and neurotransmitter receptor function are that 1) they tend to decrease the affinity of agonists for their receptors and 2) they mediate the coupling of receptors to second messenger effectors, such as adenylate cyclase (31). Guanine nucleotides have been shown to regulate the specific binding of agonists for a number of neurohormone or neurotransmitter receptors (for reviews, see Refs. 32–34). As described here and in previous studies (17, 19, 20, 22), peptides that bind to the human $CRF_{2\alpha}$ receptor protein expressed in cell lines can stimulate the production of adenylate cyclase. Thus, consistent with CRF receptors being coupled to a G protein, the addition of increasing concentrations of GTP or its nonhydrolyzable analogs Gpp(NH)p and GTP-γ-S to the incubation medium resulted in an inhibition of ^{125}I-Tyr^0-sauvagine binding in cells transfected with the human $CRF_{2\alpha}$ receptor. This effect seemed to be specific to the guanine nucleotides; similar experiments have indicated that equimolar concentrations of ATP had very little effect on sauvagine binding. Although there is no direct evidence, this modulation of the binding of ^{125}I-Tyr^0-sauvagine to the human $CRF_{2\alpha}$ receptor by guanine nucleotides suggests that this receptor exists in two affinity states for agonists coupled through a G protein to its second messenger system. Unfortunately, to date, the only ligands available for the biochemical study of these receptors have been agonists, making it very difficult to examine the proportions and affinities of high and low affinity states of these receptors. Further study will be required, possibly using labeled antagonists as tools, to characterize the affinity states of these receptors.

Recently, a novel peptide ligand was identified and characterized as having high affinity for the $CRF_{2\alpha}$ receptor. This mammalian form of urotensin, termed urocortin, was localized by urotensin I-like immunoreactivity and subsequently cloned from rat brain (35, 36). This peptide exhibits 63% identity with the fish urotensin I and 45% identity with rat CRF. Although this peptide has high affinity for the CRF_1 receptor and produces the same effects *in vitro* and *in vivo* as does CRF (35), it maintains its high affinity interaction for $CRF_{2\alpha}$ receptors similar to sauvagine and urotensin I. In addition, this molecule has high affinity for the CRF binding protein, establishing a key role in the regulation of the CRF system. Thus, this molecule likely represents one endogenous ligand for the mammalian $CRF_{2\alpha}$ receptor. Future studies will be aimed toward the identification and characterization of these and other ligands and receptors and will subsequently allow us to gain a greater awareness of the CRF system.

As demonstrated in this study, ^{125}I-Tyr^0-sauvagine was found to be a high affinity radioligand for the study of the human $CRF_{2\alpha}$ receptor. The main advantage of this ligand over other CRF-related peptides is that although it has high affinity for the CRF_1 receptor, it retains its high affinity for the human $CRF_{2\alpha}$ receptor, whereas the prior conventional ligands (oCRF and r/hCRF) have lower affinity. Furthermore, unlike urotensin I, which also retains its high affinity for the $CRF_{2\alpha}$ receptor subtype, sauvagine has relatively low affinity for the CRF binding protein, making it selective for the receptor aspect of the CRF system. The fact that studies are now demonstrating that the CRF system consists of a family of receptors that differ dramatically in structure, pharmacological profile, anatomic localization within the central nervous system, and periphery suggests that these proteins (within the scope of this system) will be differentially regulated in response to a variety of physiological challenges. Furthermore, the identification of novel endogenous ligands for these subtypes not only has led to the generation of novel hypotheses regarding CRF function but also, more importantly, has identified a tangible goal of generating selective CRF receptor antagonists as therapeutic agents that can target specific aspects of CRF-controlled behavior and physiology. These subtype-selective antagonists will allow us to pharmacologically distinguish these receptor subtypes *in vivo* and thus provide evidence for their functional significance. Future studies will focus on using tools such as ^{125}I-Tyr^0-sauvagine to identify specific and selective $CRF_{2\alpha}$ receptor antagonists in an attempt to understand the mechanisms by which CRF manifests its actions.

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Send reprint requests to: Dimitri E. Grigoriadis, Ph.D., Neurocrine Biosciences, Inc., 3050 Science Park Road, San Diego CA 92121. E-mail: dgrigoriadis@neurocrine.com