0026-895X/08/7305-1371–1380$20.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 73:1371–1380, 2008

Vol. 73, No. 5
42978/3326448
Printed in U.S.A.

Allosteric Ligands for the Corticotropin Releasing Factor Type 1 Receptor Modulate Conformational States Involved in Receptor Activation[S]

Sam R. J. Hoare, Beth A. Fleck, Raymond S. Gross, Paul D. Crowe, John P. Williams, and Dimitri E. Grigoriadis

Emerging Technologies (S.R.J.H., R.S.G, P.D.C, J.P.W, D.E.G.) and Endocrine and Metabolic (B.A.F, D.E.G.) Biomedical Research and Innovative Discovery Groups, Neurocrine Biosciences Inc., San Diego, California

Received October 26, 2007; accepted January 28, 2008

ABSTRACT

Allosteric modulators of G-protein-coupled receptors can regulate conformational states involved in receptor activation (*Mol Pharmacol* **58:**1412–1423, 2000). This hypothesis was investigated for the corticotropin-releasing factor type 1 (CRF_1) receptor using a novel series of ligands with varying allosteric effect on CRF binding (inhibition to enhancement). For the G-protein-uncoupled receptor, allosteric modulation of CRF binding was correlated with nonpeptide ligand signaling activity; inverse agonists inhibited and agonists enhanced CRF binding. These data were quantitatively consistent with a two-state equilibrium underlying the modulation of CRF binding to the G-protein-uncoupled receptor. We next investigated the allosteric effect on CRF-stimulated G-protein coupling. Ligands inhibited CRF-stimulated cAMP accumulation regardless of their effect on the G-protein-uncoupled state. The modulators reduced CRF E_{max} values, suggesting that they reduced the efficacy of a CRF-bound active state to couple to G-protein. Consistent with this hypothesis, the modulators inhibited binding to a guanine nucleotide-sensitive state. Together, the results are quantitatively consistent with a model in which 1) the receptor exists in three predominant states: an inactive state, a weakly active state, and a CRF-bound fully active state; 2) allosteric inverse agonists stabilize the inactive state, and allosteric agonists stabilize the weakly active state; and 3) antagonism of CRF signaling results from destabilization of the fully active state. These findings imply that nonpeptide ligands differentially modulate conformational states involved in CRF_1 receptor activation and suggest that different conformational states can be targeted in designing nonpeptide ligands to inhibit CRF signaling.

Nonpeptide antagonists targeting the corticotropin-releasing factor (CRF) type 1 receptor have been developed as potential treatments for anxiety and depression (Kehne and De Lombaert, 2002). The development of these ligands has been stimulated by the hypothesis that anxiety and depression are stress-related disorders (Holsboer, 2001) and by the established role of CRF as a principal regulator of the stress axis (Bale and Vale, 2004). CRF is a 41 amino acid peptide that regulates the hypothalamic-pituitary axis (Bale and Vale, 2004). CRF is secreted from the hypothalamus and stimulates the release of adrenocorticotropin from pituitary corticotropes, which in turn stimulates corticosteroid release from the adrenal medulla. CRF also acts on CRF_1 receptors in cerebral cortical and limbic nuclei regulating behavior (Smagin et al., 2001). Studies of the physiology and patho-

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.042978.
[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: CRF, corticotropin-releasing factor; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; ECD, N-terminal extracellular domain; GPCR, G-protein-coupled receptor; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; NBI 30775, 2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylaminopyrazolo[1,5-*a*]pyrimidine; NBI 34041, 2-(2,4-dichlorophenyl)-4-methyl-6-(1-propylbutyl)-7,8-dihydro-6*H*-1,3,6,8*a*-tetraaza-acenaphthylene; NBI 35965, (*S*)-6-cyclopropylmethyl-2-(2,4-dichlorophenyl)-7-ethyl-4-methyl-7,8-dihydro-6*H*-1,3,6,8*a*-tetraaza-acenaphthylene; NBI 38242, (*R*)-6-cyclopropylmethyl-2-(2,4-dichlorophenyl)-7-ethyl-4-methyl-7,8-dihydro-6*H*-1,3,6,8*a*-tetraaza-acenaphthylene; NBI 77165, (*R*)-6-phenyl-2-(2,4-dichlorophenyl)-7-ethyl-4-methyl-7,8-dihydro-6*H*-1,3,6,8*a*-tetraaza-acenaphthylene; NBI 77172, (*S*)-6-phenyl-2-(2,4-dichlorophenyl)-4-methyl-7-benzyl-7,8-dihydro-6*H*-1,3,6,8*a*-tetraaza-acenaphthylene; NBI 77173, (*R*)-6-cyclopropylmethyl-2-(2,4-dichlorophenyl)-4-methyl-7-benzyl-7,8-dihydro-6*H*-1,3,6,8a-tetraaza-acenaphthylene; NBI 77178, (*R*)-6-phenyl-2-(2,4-dichlorophenyl)-4-methyl-7-benzyl-7,8-dihydro-6*H*-1,3,6,8*a*-tetraaza-acenaphthylene; R, inactive receptor state; R*, weakly active receptor state; R**, fully active receptor state; HPLC, high-performance liquid chromatography; CHO, Chinese hamster ovary; ANOVA, analysis of variance.

physiology of the CRF system in animal models and humans implicate antagonism of central CRF_1 receptors as a potential, novel mechanism for treating anxiety, depression, and other stress-related disorders (Holsboer, 2001; Smagin et al., 2001).

Numerous selective, high-affinity, orally bioavailable nonpeptide antagonists have been developed that target the CRF_1 receptor, a class B GPCR (Kehne and De Lombaert, 2002). Prototypical compounds are efficacious in animal models of CRF- and environmentally induced stress responses (Holsboer, 2001; Smagin et al., 2001; Kehne and De Lombaert, 2002). Clinical efficacy has also been suggested in early stage human trials (Zobel et al., 2000; Ising et al., 2007). The ligands investigated to date are allosteric modulators of CRF binding (Liaw et al., 1997; Hoare et al., 2003; Zhang et al., 2003). CRF binds the CRF_1 receptor according to a two-domain model (Perrin et al., 1998; Nielsen et al., 2000; Hoare et al., 2004; Grace et al., 2007; Mesleh et al., 2007), interacting with the extracellular N-terminal domain of the receptor (ECD) with moderate affinity (Hoare et al., 2004). CRF can then interact readily with the transmembrane domain, thereby activating the receptor and intracellular signaling (stimulation of adenylyl cyclase activity) (Nielsen et al., 2000; Hoare et al., 2004). CRF binds to determinants in the extracellular loops and extracellular-proximal regions of the predicted membrane-spanning α-helices (Liaw et al., 1997; Assil-Kishawi and Abou-Samra, 2002; Dautzenberg et al., 2002; Kraetke et al., 2005). Nonpeptide antagonists bind more centrally within the transmembrane domain (Liaw et al., 1997; Hoare et al., 2006) and allosterically inhibit CRF interaction with its spatially distinct binding determinants on the transmembrane domain (Hoare et al., 2004). This allosteric inhibition blocks the CRF interaction required to activate the receptor, resulting in antagonism.

The conformational basis of this allosteric modulation of the CRF_1 receptor remains largely unknown. Multiple conformational states of the CRF_1 receptor have been inferred, differing in the mechanism of their desensitization (Perry et al., 2005) or in their ligand affinity (e.g., G-protein coupled and uncoupled states, with high and low affinity for agonists, respectively; Hoare et al., 2003). Nonpeptide ligands strongly inhibit CRF binding to the G-protein-coupled state but weakly inhibit binding to the uncoupled receptor (Hoare et al., 2003). GPCRs in general are conformationally heterogeneous, and exploiting this dynamic nature in drug discovery has received considerable recent attention (Kenakin, 2007; Kobilka and Deupi, 2007). Biophysical studies of rhodopsin, a class A GPCR, have identified multiple conformational states associated with receptor activation (Hubbell et al., 2003). Fluorescence spectroscopy applied to class A GPCRs activated by diffusible ligands has also directly demonstrated discrete conformational states that vary with respect to their capacity to activate G-protein or undergo desensitization (Ghanouni et al., 2001; Swaminath et al., 2004; Nikolaev et al., 2006; Kobilka and Deupi, 2007). Pharmacological data and modeling are consistent with multiple conformations of GPCRs with differential ability to selectively target specific downstream receptor signaling and/or desensitization pathways (Kenakin, 2007).

Conformational states involved in GPCR activation are involved in the mechanism of action of some allosteric modulators. A general model combining the allosteric ternary model of modulator action with the two-state model of receptor activation explains the mechanism of action of several allosteric ligands (Bruns and Fergus, 1990; Hall, 2000). Allosteric modulators of class C GPCRs modulate multiple conformational states involved in receptor activation (Parmentier et al., 2002). These findings prompted us to explore the hypothesis that allosteric modulation of the CRF_1 receptor by nonpeptide ligands involves regulation of conformational states involved in receptor activation and to investigate the extent to which different ligands vary in their capacity to modulate these states.

Materials and Methods

Materials. Rat/human CRF, [Tyr^0]astressin (Miranda et al., 1994), and peptide 19 (peptide number 19 of Yamada et al., 2004) were synthesized by solid-phase methodology on a Beckman Coulter 990 peptide synthesizer (Beckman Coulter, Fullerton, CA) using *t*-Boc-protected amino acids. The assembled peptide was deprotected with hydrogen fluoride and purified by preparative HPLC. The purity of the final product, assessed by analytical HPLC and mass spectrometric analysis using an ion-spray source, was >95%. The peptides were dissolved in 10 mM acetic acid/0.1% bovine serum albumin at a concentration of 1 mM and stored at −80°C. ^{125}I-[Tyr^0]astressin was synthesized using the chloramine-T method and purified by HPLC (specific activity, 2200 Ci/mol). ^{125}I-[Tyr^0] sauvagine was from PerkinElmer Life and Analytical Sciences (Waltham, MA). Nonpeptide ligands were synthesized as described in Gross et al. (2005) for NBI 35965, NBI 38242, and NBI 34041. NBI 77172 was synthesized as described in Gross et al. (2005) using the appropriate intermediate used to prepare compound 12g of this reference and alkylating using benzyl bromide. NBI 77173 and NBI 77178 were synthesized using the *R*-enantiomer of the appropriate intermediate, as described in Gross et al. (2005), and alkylating with bromomethylcyclopropane and benzyl bromide, respectively. NBI 30775 was synthesized as described previously (Chen et al., 2004). Nonpeptide ligands were dissolved in 100% dimethyl sulfoxide at a concentration of 10 mM and stored at −20°C. [^{3}H]NBI 35965 was prepared as described previously (Hoare et al., 2006). G418 (Geneticin), Dulbecco's phosphate-buffered saline (DPBS), and cell culture supplies were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from HyClone (Logan, UT). Pertussis toxin was from EMD Biosciences (San Diego, CA). AtT-20/D16v-F2 cells were obtained from American Type Culture Collection (Manassas, VA).

Cell Culture and Preparation of Cell Membranes. A CHO cell line was used for the detection of constitutive receptor activity in whole-cell cAMP assays (St-Denis et al., 2005) that highly overexpresses the human CRF_1 receptor (^{125}I-astressin B_{max} = 100 ± 20 pmol/mg). This cell line, referred to here as CHO-CRF_1, was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 250 μg/ml G418. AtT20 cells (Litvin et al., 1984) were grown in DMEM supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 IU/ml penicillin, and 50 μg/ml streptomycin. For radioligand binding assays, a Flp-In-CHO cell line expressing the human CRF_1 receptor was used that has been extensively characterized in radioligand binding experiments, with a B_{max} value of 28 ± 6 pmol/mg membrane protein (Hoare et al., 2006). Cell membranes were prepared from these cells using nitrogen cavitation and differential centrifugation as described previously (Hoare et al., 2003).

cAMP Accumulation Assays. Accumulation of cAMP was measured in CHO-CRF_1 and AtT20 cells plated 18 h before the assay into 96-well plates [poly(lysine) coated for AtT20 cells]. In initial experiments on CHO-CRF_1 cells, a bell-shaped CRF concentration-dependence curve was observed. The descending, inhibitory component of this curve was blocked by including pertussis toxin when plating the cells, consistent with coupling to inhibitory G-proteins. As a conse-

quence, CHO-CRF$_1$ cells were plated with 100 ng/ml pertussis toxin 18 h before the assay. CHO-CRF$_1$ cells were plated at a density of 5000 cells/well, and AtT20 cells were plated in the absence of pertussis toxin at a cell density of 2.7 to 4 × 10^4 cells/well. For the assay, medium was removed, and the cells were washed with 150 μl of DPBS. After aspiration of DPBS, 75 μl of cAMP assay buffer was added to each well (DMEM without phenol red, supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 1 mM 3-isobutyl-1-methylxanthine). Ligands were then added in a volume of 25 μl. In experiments in which more than one compound was included in the assay (receptor ligand with forskolin or peptide with nonpeptide ligand), compounds were diluted together before addition to the assay so that cells were exposed simultaneously to the two ligands. Cells were incubated for 30 min at 37°C in 5% CO_2. cAMP was measured by chemiluminescent immunoassay (Tropix, Bedford, MA).

Radioligand Binding Assays. Binding assays were performed in membranes from Flp-In-CHO cells expressing the CRF$_1$ receptor. Assays were set up in assay buffer, composed of DPBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 138 mM NaCl), supplemented with 10 mM $MgCl_2$, and 2 mM EGTA, pH adjusted to 7.4 with NaOH. All assays were incubated in low-binding 96-well plates (Corning, Palo Alto, CA) for 2 h at 22°C, followed by the separation of bound and free radioligand by rapid filtration and radioactivity counting as described previously (Hoare et al., 2003). The wash buffer used in the filtration step was DPBS for [^{3}H]NBI 35965 and DPBS supplemented with 0.01% Triton X-100 for ^{125}I-astressin and ^{125}I-sauvagine. Total radioligand binding was less than 20% of the total radioligand concentration in the assay.

In the first series of binding experiments, the effect of nonpeptide ligand on CRF binding to the G-protein-uncoupled state of the receptor was measured using a method described previously (Hoare et al., 2003). CRF binding to the uncoupled state was measured by displacement of ^{125}I-astressin binding to cell membranes in the presence of 30 μM GTPγS using single determinations at each CRF concentration tested. CRF binding was measured alone and in the presence of seven concentrations of nonpeptide ligand. The reagents were added in the following order and volume: 25 μl of GTPγS, 25 μl of ^{125}I-astressin, 25 μl of CRF, 50 μl of nonpeptide ligand, and 75 μl of membranes. The concentration of ^{125}I-astressin varied between 63 and 110 pM, and the amount of membrane used was 70 to 150 ng/well. Saturation analysis indicated ^{125}I-astressin bound to a single affinity state, with a K_d value of 31 pM and B_{max} value of 28 pmol/mg.

In the second series of binding experiments, the affinity of nonpeptide ligands was measured by inhibition of [^{3}H]NBI 35965 binding. Binding to the G-protein-uncoupled state of the receptor was measured in cell membranes by including 30 μM GTPγS. Binding of each concentration of nonpeptide ligand was measured in duplicate. The reagents were added in the following order and volume: 25 μl of GTPγS, 50 μl of [^{3}H]NBI 35965, 50 μl of nonpeptide ligand, and 75 μl of membranes. The concentration of [^{3}H]NBI 35965 varied between 1.8 and 5.5 nM, and the amount of membrane used was 2.0 to 5.0 μg/well. Saturation analysis indicated [^{3}H]NBI 35965 bound to a single affinity state, with a K_d value of 480 pM and B_{max} value of 26 pmol/mg.

In the third series of binding experiments, the effect of nonpeptide ligands on ^{125}I-sauvagine binding was measured in the absence and presence of 30 μM GTPγS. Binding for each condition was measured in duplicate. The reagents were added in the following order and volume: 25 μl of ^{125}I-sauvagine, 25 μl of GTPγS or buffer alone, 50 μl of nonpeptide ligand, and 100 μl of membranes. The concentration of ^{125}I-sauvagine varied between 56 and 63 pM, and the amount of membrane used was 1.0 to 2.0 μg/well. Saturation analysis indicated a high-affinity state sensitive to GTPγS (K_d value of 19 pM, B_{max} value of 1.8 pmol/mg) and a lower-affinity state insensitive to GTPγS, for which the affinity and B_{max} value could not be reliably determined because saturation could not be reached at the highest concentration tested (3 nM). To assess the effect of nonpeptide ligand on GTPγS-sensitive ^{125}I-sauvagine binding, specific binding in the presence of GTPγS was subtracted from specific binding in the absence of GTPγS for each concentration of nonpeptide ligand tested. Specific binding was calculated by subtracting nonspecific binding (the lower asymptote of a CRF displacement curve, included as a control in each experiment).

Data Analysis. The allosteric effect of nonpeptide ligands on CRF binding (Fig. 2) was analyzed using eq. 1:

$$[RL^*] + [NRL^*] = \frac{[R_{TOT}]([L^*]/K_{L^*})(1 + C_{L^*}([N]/K_N))}{1 + ([L^*]/K_{L^*})(1 + C_{L^*}([N]/K_N)) + ([L]/K_L)(1 + C_L([N]/K_N)) + ([N]/K_N)} + NSB \quad (1)$$

TABLE 1

Nonpeptide ligand affinity and cooperativity with peptide ligands

Nonpeptide ligand binding activity was measured using two methods: 1) the observed cooperativity between nonpeptide ligand and CRF (C_{CRF}) and nonpeptide ligand affinity ($K_{nonpeptide}$) were measured for the G-protein-uncoupled receptor state (^{125}I-astressin vs CRF + GTPγS), as described in Fig. 2. Data are mean ± S.E.M. (n = 3–4). −log K_{CRF} was not significantly different when measured in assays of the different nonpeptide ligands ($P > 0.05$, single-factor ANOVA, mean of all values of 7.20 ± 0.04, n = 27, K_{CRF} = 64 nM). The ^{125}I-astressin concentration was 63 to 100 pM and K_d was 31 pM. 2) nonpeptide ligand affinity for the G-protein-uncoupled state was measured more directly by displacement of labeled nonpeptide ligand binding ([^{3}H]NBI 35965 + GTPγS) as described under *Materials and Methods*. Data are mean ± S.E.M. (n = 3–14). −log $K_{nonpeptide}$ was significantly different between the two methods (P = 0.0034, two-factor ANOVA), but the Bonferroni post test indicated a significant difference between the methods only for NBI 38242 ($P < 0.01$) and NBI 30775 ($P < 0.05$, 3.2- and 2.9-fold differences, respectively). Differences of C_{CRF} between nonpeptide ligands were tested statistically using single-factor ANOVA ($P < 0.0001$) with the Newman-Keuls post test indicating all values were significantly different from one another, except for two pairs, NBI 30775 and NBI 34041, and NBI 77173 and NBI 77165.

Ligand	^{125}I-Astressin vs CRF								[^{3}H]NBI 35965	
	log $C_{astressin}$	$C_{astressin}$	log C_{CRF}	C_{CRF}	−log K_{CRF}	K_{CRF}	−log $K_{nonpeptide}$	$K_{nonpeptide}$	−log $K_{nonpeptide}$	$K_{nonpeptide}$
						nM		*nM*		*nM*
NBI 30775	−0.30 ± 0.02	0.50	−0.96 ± 0.07	0.11	7.41 ± 0.11	39	9.06 ± 0.05	0.87	8.60 ± 0.06	2.5
NBI 34041	−0.42 ± 0.05	0.38	−0.76 ± 0.06	0.18	7.11 ± 0.07	77	8.68 ± 0.33	2.1	8.62 ± 0.04	2.4
NBI 35965	−0.18 ± 0.03	0.66	−0.70 ± 0.05	0.20	7.23 ± 0.08	59	8.72 ± 0.10	1.9	8.69 ± 0.04	2.1
NBI 38242	−0.28 ± 0.03	0.52	−0.48 ± 0.06	0.33	6.98 ± 0.14	100	6.72 ± 0.10	190	7.22 ± 0.05	60
NBI 77173	−0.24 ± 0.04	0.58	0.96 ± 0.04	9.0	7.28 ± 0.07	53	6.10 ± 0.11	800	6.55 ± 0.06	280
NBI 77165	0.27 ± 0.04	1.9	0.91 ± 0.10	8.2	6.94 ± 0.01	115	6.14 ± 0.04	720	6.57 ± 0.19	270
NBI 77178	0.12 ± 0.07	1.3	1.49 ± 0.04	31	7.26 ± 0.07	55	5.66 ± 0.06	2,200	6.14 ± 0.02	730
NBI 77172	−0.10 ± 0.01	0.79	0.08 ± 0.05	1.2	7.26 ± 0.14	55		N.D.[a]	7.08 ± 0.05	84

N.D., not determined.

[a] The cooperative effect of NBI 77172 on peptide binding was too small to reproducibly determine nonpeptide ligand affinity, so in the fitting procedure, $K_{nonpeptide}$ was fixed at the value measured by displacement of [^{3}H]NBI 35965 binding.

where R is receptor, L* is peptide radioligand (^{125}I-astressin), L is unlabeled peptide ligand (CRF), N is nonpeptide allosteric modulator, K_{L*}, K_L, and K_N are the corresponding equilibrium dissociation constants, C_{L*} is the observed cooperativity between nonpeptide and radioligand (denoted $C_{astressin}$ in Table 1), C_L is the observed cooperativity between nonpeptide and unlabeled peptide ligand (denoted C_{CRF} in Table 1), [R_{TOT}] is the total receptor concentration, and NSB is nonspecific binding. This equation was derived as described previously (Hoare et al., 2003). Total binding data (cpm) were fit to this equation using SigmaPlot 8.0 (SPSS Science, Chicago, IL) with the concentration of CRF and nonpeptide ligand as independent variables. NSB was assumed to be equal for all concentrations of nonpeptide ligand and was fitted as a common lower asymptote of the displacement curves.

Inhibition of [^{3}H]NBI 35965 and inhibition of GTPγS-sensitive ^{125}I-sauvagine binding by nonpeptide ligand was fit by a single-state inhibition equation using Prism 4.01 (GraphPad Software Inc., San Diego, CA), and the IC_{50} value was converted to K_i using the method of Cheng and Prusoff (1973). A sigmoid concentration-response equation (slope factor fixed at unity) was fit to cAMP accumulation data using Prism 4.01. Initial analyses using a four parameter-logistic equation indicated the slope factor was close to unity in all assays (between 0.8 and 1.2).

Results

The aim of this study was to test the hypothesis that allosteric modulation by nonpeptide ligands involves the modulation of conformational states associated with CRF_1 receptor activation. Previous studies indicate differences of allosteric effect by nonpeptide ligand depending on the conformational state of the CRF_1 receptor, with modest allosteric inhibition at the receptor uncoupled from G-protein and strong inhibition at the G-protein-coupled receptor (Hoare et al., 2003). We first investigated the allosteric mechanism at the uncoupled receptor.

Discovery of a Series of Nonpeptide Ligands with a Range of Allosteric Activity. To facilitate this investigation we used a series of nonpeptide ligands displaying a range of allosteric activity on CRF binding. Allosteric modulation of CRF binding was measured using a triple-ligand assay (Hoare et al., 2003). ^{125}I-Astressin was used as the radioligand. Binding of this radioligand, predominantly to the ECD of the CRF_1 receptor (Perrin et al., 1998), is not appreciably inhibited by nonpeptide ligands. ^{125}I-Astressin binding is fully inhibited by CRF (Fig. 2), which binds the ECD and transmembrane domain of the receptor (Hoare et al., 2004). The allosteric effect of nonpeptide ligand can then be evaluated by measuring its effect on the CRF inhibition curve for displacing ^{125}I-astressin binding. Finally, a high concentration of GTPγS was included in the assay to uncouple the receptor from G-protein, providing a read-out of the allosteric effect on receptor uncoupled from G-protein.

The prototypical antagonist NBI 35965 (Fig. 1C) allosterically inhibited CRF binding, evident as a rightward shift of the CRF inhibition curve (Fig. 2A). Global curve-fitting using eq. 1 (as described under *Materials and Methods*) indicated an observed cooperativity factor (C_{CRF}) of 0.20 (Table 1), indicating that NBI 35965 binding to the CRF_1 receptor reduced the affinity of CRF by 5-fold (in agreement with previous data; Hoare et al., 2003). NBI 35965 possesses a chiral center in the top region (Fig. 1C). Reversing the stereochemical configuration from *S* to *R* (NBI 38242; Fig. 1C) reduced the allosteric inhibition effect (C_{CRF} of 0.33, indicating a 3-fold reduction of CRF affinity; Fig. 2B and Table 1). We further explored the structure-activity relationship of the *R*-configuration (Fig. 1C). It is striking that replacing the 7-ethyl group with phenyl (NBI 77173) changed the allosteric effect from inhibition to enhancement of CRF binding (C_{CRF} of 9.0, indicating a 9-fold increase of CRF affinity; Table 1). This effect was evident as a leftward shift of the CRF inhibition curve (Fig. 2C). Furthermore, additional replacement of the 6-cyclopropyl group with benzyl (NBI 77178) increased the allosteric enhancement (C_{CRF} of 31, indicating a 31-fold increase of CRF affinity; Fig. 2D and Table 1). The structure-activity relationship of this series was explored with two additional compounds (NBI 77165 and NBI 77172; Fig. 1C) as summarized in Table 1. In addition, two compounds tested clinically acted as allosteric inhibitors (NBI 30775 and NBI 34041; Fig. 1, A and B, and Table 1). In summary, a series of nonpeptide ligands was identified that displayed a considerable range of allosteric effect on the G-protein-uncoupled CRF_1 receptor, ranging from inhibition to enhancement of CRF binding.

Correlation between Allosteric Modulation of CRF Binding to the Uncoupled Receptor and Signaling Activity of Nonpeptide Ligands. A previously described mechanism of allosteric modulation invokes active and inactive states of GPCRs (Hall, 2000). In this model, receptor is in equilibrium between inactive and active states (R and R*, respectively). A full agonist (e.g., CRF) binds preferentially to R* over R. Allosteric inhibition of agonist binding results from modulator-stabilizing R, which binds agonist with lower affinity, whereas allosteric enhancement results from modulator stabilizing R*, which binds agonist with higher affinity. Within the two-state model, stabilization of R* versus R can be evaluated using the intrinsic activity (E_{max}) of the ligand; redistribution toward R* results in agonism and reciprocally shifting the distribution toward R results in inverse agonism. We therefore tested the hypothesis that allosteric modulation of CRF binding results from changes of an R/R* equilibrium by measuring the intrinsic signaling activity of nonpeptide modulators (in the absence of CRF) and comparing the intrinsic activity values with those for allosteric modulation.

A highly sensitive signaling system was developed to detect efficacy of nonpeptide ligands (see Supplemental Infor-

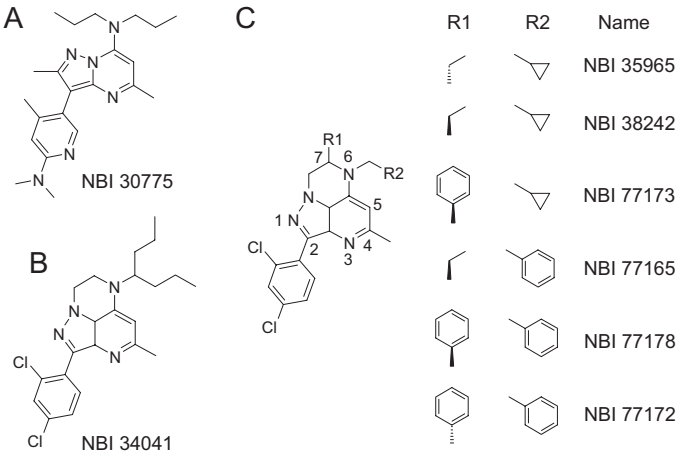

Fig. 1. Chemical structure of nonpeptide ligands used in this study. A, NBI 30775 (Chen et al., 2004). B, NBI 34041 (Gross et al., 2005). C, NBI 35965 series, showing "top" region modifications (Gross et al., 2005).

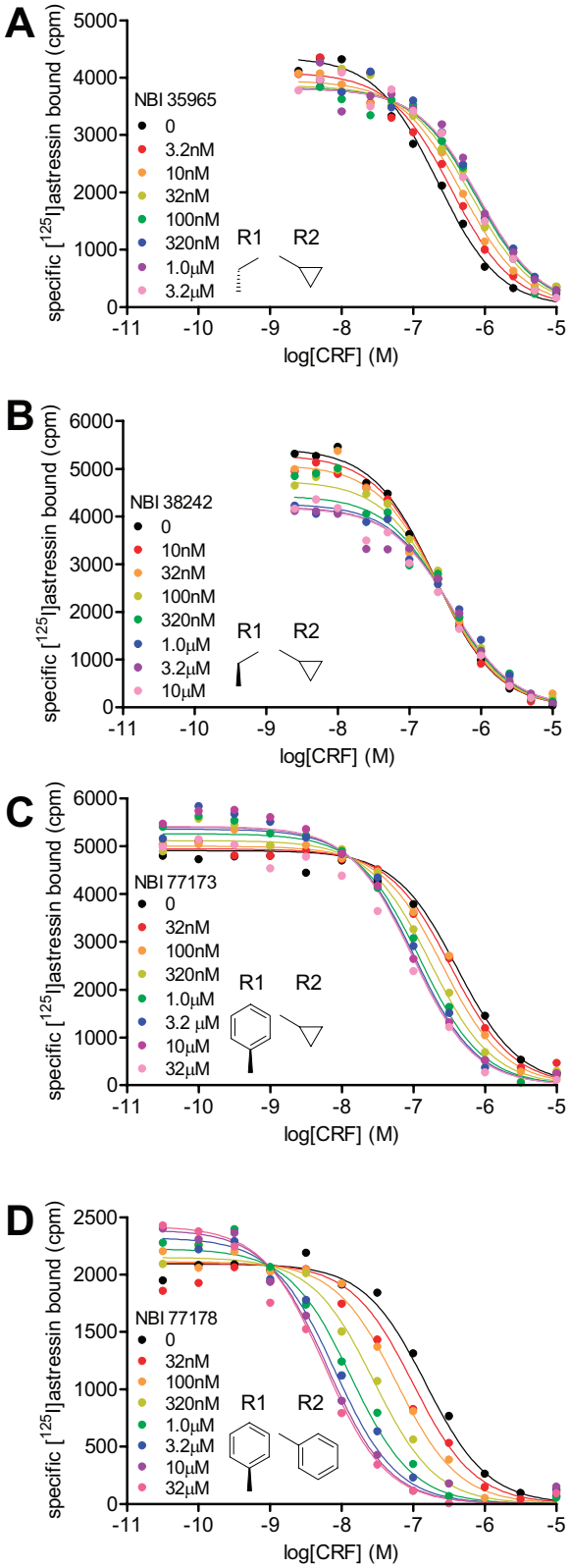

Fig. 2. Allosteric modulation of CRF binding to the G-protein-uncoupled CRF$_1$ receptor. CRF binding was measured by its ability to displace the radiolabeled antagonist ^{125}I-astressin from the CRF$_1$ receptor, which was uncoupled from G-protein using GTPγS. CRF binding was measured in the absence of nonpeptide ligand and in the presence of seven concentrations of nonpeptide ligand (A, NBI 35965; B, NBI 38242; C, NBI 77173; D, NBI 77178), and the data were analyzed globally with eq. 1, which assumes allosteric modulation of peptide binding (CRF and astressin) by nonpeptide ligand (see *Materials and Methods*). The r^2 value for the curve fit was between 0.95 and 0.99 for all compounds tested. The fitted parameter values are given in Table 1. R1 and R2 refer to the chemical structure in Fig. 1C. Data are from representative experiments performed three to four times.

mation). The signaling response (stimulation of cAMP accumulation) was highly amplified by receptor overexpression (100 pmol/mg). Despite the very high level of receptor expression, constitutive activity could not be reliably detected, indicating very low constitutive activity of the CRF$_1$ receptor. Constitutive activity could be detected using forskolin to amplify the response (see Supplemental Information). The conditions used allowed for the detection of constitutive receptor activity, small partial agonist responses, and inverse agonism (Supplemental Fig. S1C). The prototypical antagonist NBI 35965, an allosteric inhibitor (C_{CRF} of 0.20), acted as an inverse agonist, reducing basal activity by 77% (Fig. 3 and Table 2). The allosteric inhibitors NBI 30775 and NBI 34041 (Table 1) also acted as inverse agonists (Table 2 and Supplemental Fig. S1C). In contrast, the allosteric enhancers NBI 77173 and NBI 77178 acted as agonists, with efficacy approaching that of CRF (Fig. 3, Table 2). This finding suggests a correlation between allosteric modulation and signaling efficacy. In Fig. 4, this correlation was quantified using additional ligands within the same series (NBI 38242, NBI 77165, and NBI 77172; Table 2). Linear regression indicated a significant correlation between allosteric modulation of CRF binding (C_{CRF}) and intrinsic activity of nonpeptide ligands ($r^2 = 0.96$, $p < 0.0001$; broken line on Fig. 4). The data were also well fit by a sigmoid curve ($r^2 = 0.98$) with a slope

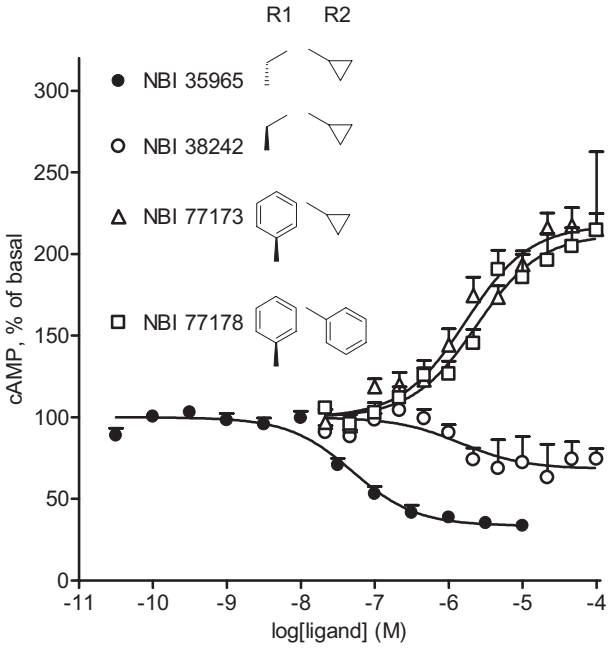

Fig. 3. cAMP accumulation in CHO-CRF$_1$ cells in response to nonpeptide ligands. The cAMP response was measured in cells pretreated with pertussis toxin (to minimize inhibition of cAMP accumulation) and treated in the assay with 1 μM forskolin (to amplify constitutive and ligand-stimulated receptor activity). Data points are pooled mean ± S.E.M. of determinations from three to seven experiments. The data have been normalized to the basal activity in the absence of ligand and in the presence of 1 μM forskolin. This value was measured as the "no ligand" asymptote of the curve fit. R1 and R2 refer to the chemical structure in Fig. 1C.

TABLE 2

cAMP accumulation in CHO-CRF_1 cells treated with 1 μM forskolin in response to peptide and nonpeptide ligands

The cAMP response was measured in cells pretreated with pertussis toxin (to minimize inhibition of cAMP accumulation) and treated in the assay with 1 μM forskolin (to amplify constitutive and ligand-stimulated receptor activity). Data have been normalized to basal activity (see legend to Fig. 3). See Supplementary Fig. S1C for peptide ligands. Data are mean ± S.E.M., n = 3 to 14. For nonpeptide ligands, single-factor ANOVA indicated E_{max} values were significantly different, with the post hoc Newman-Keuls test indicating significant difference between the following groups: NBI 34041, NBI 30775, NBI 35965, NBI 38242 < NBI 77172 < NBI 77173, NBI 77165, and NBI 77178.

Ligand	E_{max}	$-\log EC_{50}$	EC_{50}
	% basal activity		*nM*
CRF	270 ± 15	11.43 ± 0.09	3.7 pM
Astressin	180 ± 10	8.61 ± 0.26	2.5 nM
Peptide 19	88 ± 10	N.D.	N.D.
NBI 30775	33 ± 4	7.35 ± 0.05	45 nM
NBI 34041	18 ± 5	7.44 ± 0.10	36 nM
NBI 35965	33 ± 3	7.29 ± 0.11	51 nM
NBI 38242	75 ± 10	6.12 ± 0.35	750 nM
NBI 77173	210 ± 20	5.62 ± 0.12	2.4 μM
NBI 77165	210 ± 10	5.62 ± 0.11	2.4 μM
NBI 77178	230 ± 20	5.50 ± 0.24	3.1 μM
NBI 77172	150 ± 10	5.56 ± 0.29	2.7 μM

N.D., not determined.

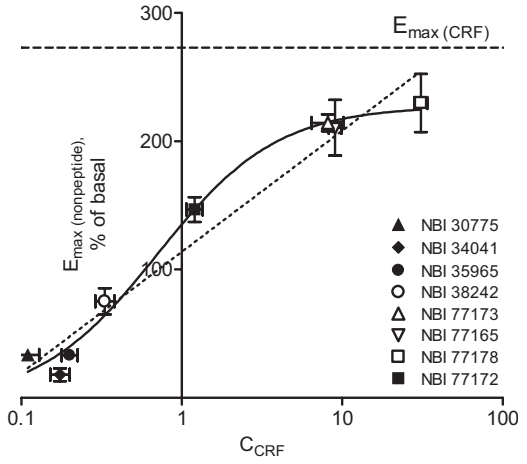

Fig. 4. Correlation between allosteric modulation of CRF binding and signaling efficacy of nonpeptide ligands. Signaling efficacy is given as the maximal percentage of stimulation of basal cAMP accumulation in CHO-CRF_1 cells (Fig. 3 and Table 2), and allosteric modulation of CRF binding (G-protein-uncoupled receptor) is represented as the observed cooperativity factor C_{CRF} (Fig. 2 and Table 1). The broken line is a linear regression fit, and the solid line is the best fit by a four parameter-logistic equation.

factor close to unity (1.2) and a y-intercept close to 100% (140%; solid line in Fig. 4). This correlation suggests that allosteric modulation of CRF binding to the uncoupled receptor involves changes of receptor states involved in receptor activation, consistent with an inactive/active state equilibrium of the uncoupled receptor (Hall, 2000). In Supplemental Information, simulated data are used to demonstrate that such a model is quantitatively consistent with the correlation in Fig. 4. The simulation predicts a sigmoidal relationship between C_{CRF} and E_{max}, with a slope factor of unity and a y-intercept of 100% (Fig. S2).

Allosteric Modulation of CRF-Stimulated G-Protein-Coupling by Nonpeptide Ligands. At the G-protein-uncoupled receptor, our findings are consistent with nonpeptide ligands modulating an R/R* equilibrium in allosterically modulating CRF binding. We next investigated how nonpeptide ligands modulate G-protein-coupling stimulated by CRF. We tested whether the two-state model was sufficient to account for the modulation of G-protein coupling. According to this model, ligands that allosterically inhibit CRF binding by enriching R (e.g., NBI 35965) inhibit CRF signaling, and modulators that enhance CRF binding by enriching R* (e.g., NBI 77173) enhance CRF signaling. CRF signaling (stimulation of cAMP accumulation) was measured in a system with low receptor expression. At low receptor expression, allosteric modulation of agonist efficacy can be readily detected as a change of E_{max}, whereas at high levels of expression changes of efficacy are manifest as changes of EC_{50} values, from which it is more complicated to ascertain the effect on agonist efficacy. AtT20 cells were used (Litvin et al., 1984), which are mouse corticotrope cells that endogenously express the CRF_1 receptor (100 fmol/mg). In AtT20 cells, CRF stimulated cAMP accumulation with an EC_{50} value of 2.2 nM (Fig. 5). In the absence of CRF, none of the nonpeptide modulators tested in this study affected cAMP accumulation in AtT20 cells (data not shown), in contrast to CHO-CRF_1 cells (Fig. 3). This difference is probably due to the high level of signal amplification via the overexpressed receptor in CHO-CRF_1 cells, amplifying responses that are not detectable via the endogenously expressed receptor in AtT20 cells.

A ligand that allosterically inhibited CRF binding to the G-protein-uncoupled receptor (NBI 35965) antagonized the CRF response in AtT20 cells (Fig. 5A). We were surprised to find that a ligand that allosterically enhanced CRF binding

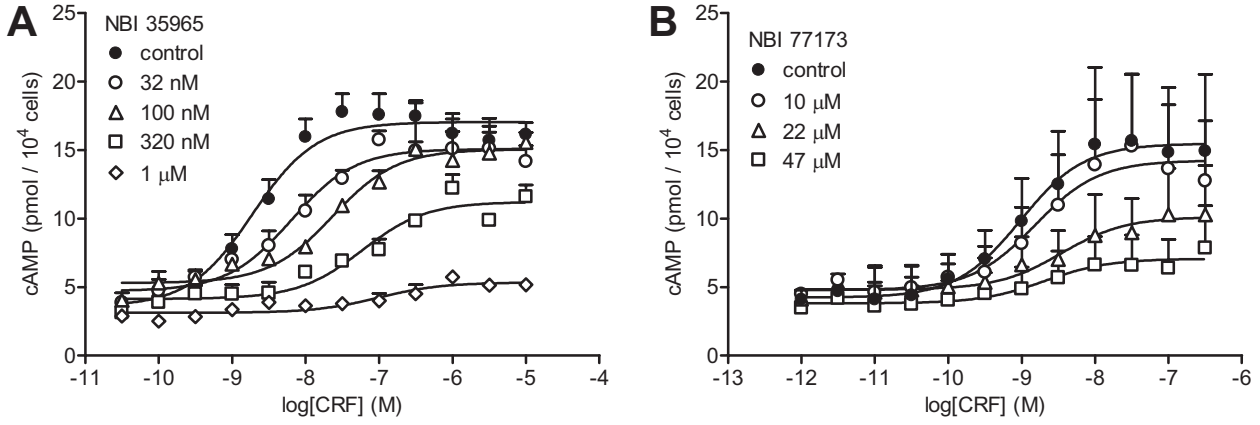

Fig. 5. Antagonism of CRF-stimulated cAMP accumulation in AtT20 cells. Antagonism by NBI 35965 (A) and NBI 77173 (B) was measured and analyzed as described under *Materials and Methods*. Data are pooled from three experiments.

to the uncoupled receptor (NBI 77173) also inhibited the CRF response (Fig. 5B). A simple two-state model of modulator action is not sufficient to explain this difference, indicating that the mechanism underlying modulation of CRF-stimulated G-protein coupling is distinct from that underlying modulation of CRF binding to the uncoupled state. Both NBI 35965 and NBI 77173 reduced the E_{max} value of CRF. This finding suggests that the nonpeptide ligands reduce the G-protein coupling efficacy of a CRF-bound state.

To investigate this possibility, the effect of nonpeptide ligands on the G-protein-coupled state was evaluated in a radioligand binding assay. A G-protein-coupled state of the CRF_1 receptor was pharmacologically isolated using ^{125}I-sauvagine, an amphibian analog of CRF (Montecucchi and Henschen, 1981). This peptide shares critical amino acid sequence determinants with CRF that are responsible for CRF_1 receptor binding and activation (Montecucchi and Henschen, 1981). Binding of ^{125}I-sauvagine is substantially inhibited by guanine nucleotide, which binds the G-protein α-subunit, implying that this binding increment represents receptor in complex with G-protein. In the current study, GTPγS inhibited ^{125}I-sauvagine binding to the CRF_1 receptor in CHO-CRF_1 cell membranes by 88% (Fig. 6). The effect of nonpeptide ligand on ^{125}I-sauvagine binding was measured in the absence and presence of GTPγS. GTPγS-sensitive ^{125}I-sauvagine binding was then calculated by subtracting the level of ^{125}I-sauvagine binding in the presence of GTPγS from the level in the absence of the nucleotide (Fig. 6).

NBI 77173 fully inhibited GTPγS-sensitive ^{125}I-sauvagine binding (Fig. 6C), in marked contrast to the enhancement of CRF binding observed on the uncoupled CRF_1 receptor (Fig. 2). It was necessary to subtract the ^{125}I-sauvagine binding increment in the presence of GTPγS to determine that NBI 77173 fully inhibited GTPγS-sensitive ^{125}I-sauvagine binding; NBI 77173 slightly enhanced ^{125}I-sauvagine binding in the presence of GTPγS ("GTPγS-insensitive" binding; Fig. 6 and Table 3). This effect was particularly pronounced for NBI 77178 (Fig. 6D and Table 3). This finding is potentially consistent with these nonpeptide ligands enhancing binding to the CRF_1 receptor uncoupled from G-protein. NBI 35965 fully inhibited GTPγS-sensitive ^{125}I-sauvagine binding to the CRF_1 receptor and inhibited GTPγS-insensitive binding (Fig. 6A and Table 3). All of the other nonpeptide ligands used in this study were evaluated, and all ligands inhibited GTPγS-sensitive ^{125}I-sauvagine binding (Fig. 6 and Table 3). This finding is consistent with nonpeptide ligands reducing the ability of a sauvagine-bound receptor state to couple to G-protein.

In summary, nonpeptide ligands attenuated CRF-stimulated G-protein coupling (recorded as cAMP accumulation) and inhibited ^{125}I-sauvagine binding to the G-protein-coupled state. These findings are potentially consistent with the nonpeptide ligands reducing the efficacy of a peptide agonist-bound state to couple to G-protein. To further test this hypothesis, the two-state model was extended to account for G-protein coupling and subsequent second-messenger signaling. The model assumes that the CRF-bound active state (R**L) (see Scheme 1 in Supplemental Data) has a higher efficacy for stimulating signaling than the free active state (R*), that nonpeptide binding to R* does not change its signaling efficacy, and that nonpeptide binding to R**L reduces the signaling efficacy of the receptor back down to that of R*.

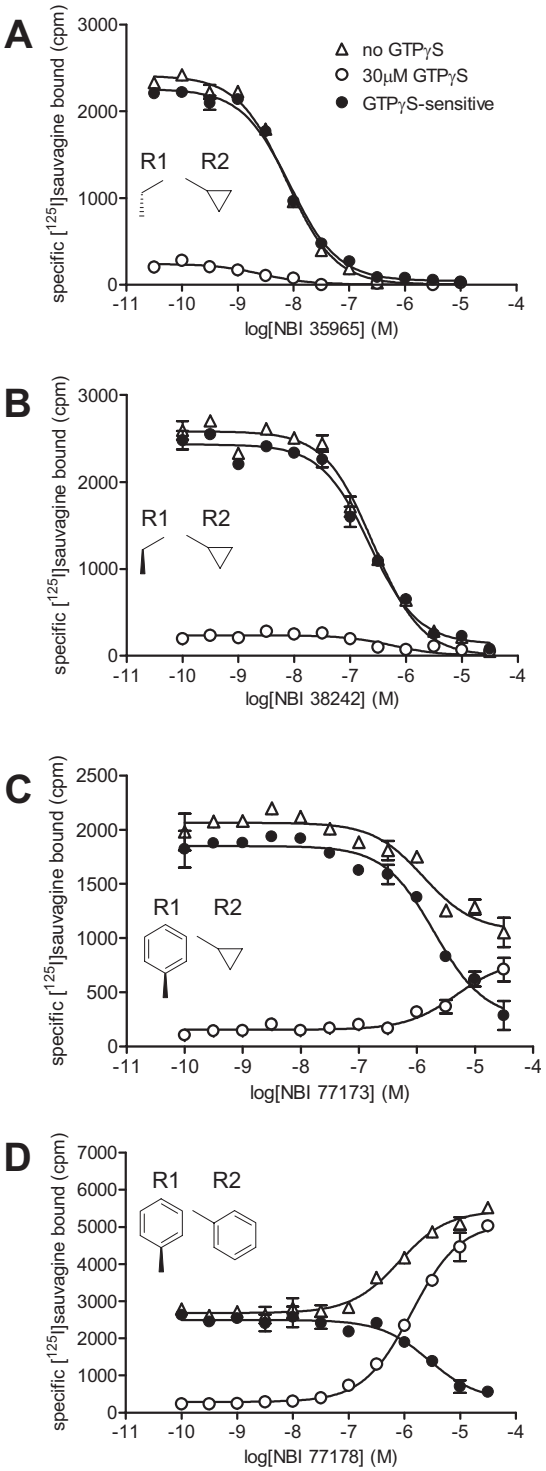

Fig. 6. Inhibition of ^{125}I-sauvagine binding to the G-protein-coupled CRF_1 receptor. This state of the receptor was pharmacologically isolated as the increment of ^{125}I-sauvagine binding which is sensitive to the guanine nucleotide GTPγS. "GTPγS-sensitive" binding was determined by subtracting specific binding in the presence of GTPγS (30 μM GTPγS) from specific binding in the absence of the nucleotide (no GTPγS). A, NBI 35965; B, NBI 38242; C, NBI 77173; D, NBI 77178. Curves are fits by a sigmoid concentration-dependence equation (slope factor fixed to unity). Data are from representative experiments performed three times. R1 and R2 refer to the chemical structure in Fig. 1C.

This model simulates well the antagonist effects of NBI 35965 and NBI 77173 on CRF-stimulated cAMP accumulation in AtT20 cells (Supplemental Fig. S2) and accounts for the ability of nonpeptide ligands that enhance CRF binding to the uncoupled receptor to inhibit binding to the G-protein-coupled state (see Supplemental Information).

Discussion

The aim of this study was to determine whether the allosteric effects of nonpeptide ligands on CRF binding and signaling involved conformational states associated with receptor activation. For the G-protein-uncoupled receptor, nonpeptide ligand modulation of CRF binding could be accounted for by a two-state allosteric model (Hall, 2000), in which allosteric inhibition of CRF binding results from stabilization of R (manifest as inverse agonism of the nonpeptide ligand), and allosteric enhancement results from enrichment of R* (manifest as partial agonism). The nonpeptide ligands antagonized CRF-stimulated G-protein coupling and inhibited ^{125}I-sauvagine binding to the G-protein-coupled state. These effects were observed regardless of the activity at the uncoupled state, suggesting a distinct mechanism of action on G-protein coupling, potentially a reduction of the G-protein coupling efficacy of a CRF-bound state. These hypotheses were incorporated into the model illustrated in Supplemental Scheme S1. This model assumes that the receptor exists in three predominant functional states: an inactive state (R); a weakly active state that signals detectably only when the receptor is overexpressed (R*); and a fully active state produced by binding of CRF that signals efficiently (R**). In Supplemental Information, this model is shown to be quantitatively consistent with the pharmacological effects observed in this study, including: constitutive activity; inverse agonism and partial agonism for the overexpressed receptor in CHO cells, but no detectable constitutive activity for the receptor expressed endogenously in AtT20 cells; reduced CRF E_{max} value by NBI 35965 and NBI 77173; increase of CRF EC_{50} by NBI 35965 but not by NBI 77173 (Fig. S2); and the sigmoidal relationship between nonpeptide ligand E_{max} and cooperativity with CRF (Fig. S3).

The different functional states in the model suggest distinct conformations of the CRF_1 receptor involved in receptor activation. The physical basis of CRF_1 receptor conformation requires more direct examination. For the β_2- and α_2-adrenergic receptors, distinct receptor conformations have been demonstrated directly that are produced by the binding of different ligands (particularly partial versus full agonist bound conformations) (Ghanouni et al., 2001; Nikolaev et al., 2006). These different conformations have been proposed to represent conformational steps along a sequential receptor activation pathway (Swaminath et al., 2004). Direct examination of CRF_1 receptor conformation could reveal that the weakly active state of the CRF_1 receptor represents an intermediate conformation along the path to the fully active state promoted by the full agonist CRF. In addition, examining CRF_1 receptor conformation could reveal conformational effects of nonpeptide ligands beyond the resolution of the indirect pharmacological methods used in this study.

In the proposed model, antagonism can result from two distinct mechanisms. NBI 35965 attenuates signaling by stabilizing the inactive state (mechanism 1), whereas NBI 77173 antagonizes CRF signaling by enriching the weakly active state that does not signal detectably for the endogenously expressed receptor in AtT20 cells (mechanism 2). The implication that NBI 35965 and NBI 77173 stabilize different conformational states could be highly useful in further development of nonpeptide ligands for the CRF_1 receptor. The ligands identified to date almost universally conform to a common topology of chemical structure (Kehne and De Lombaert, 2002). Targeting a different conformational state, such as the weakly active state in mechanism 2, could enable the identification and optimization of ligands with novel chemical structure. The different pharmacological mechanisms of NBI 35965 and NBI 77173 could also be relevant to the future development of nonpeptide ligands. Although there was little difference in the short-term antagonism of CRF signaling (Fig. 5), the different intrinsic activities of these two ligands could be significant in CRF_1 receptor regulation in the long-term exposure typical in antidepressant therapy. Different mechanisms of GPCR regulation result from treatment with ligands that stabilize different conformational states (Kenakin, 2007), and for the CRF_1 receptor, different mechanisms of receptor internalization have been suggested to result from distinct conformational states (Perry et al., 2005).

In Fig. 7, the conformational model of Supplemental

TABLE 3

Effect of nonpeptide ligand on GTPγS-sensitive and insensitive ^{125}I-sauvagine binding to the CRF_1 receptor

The effect of nonpeptide ligand on the G-protein-coupled state of the CRF_1 receptor was assessed by measuring GTPγS-sensitive ^{125}I-sauvagine binding, as described in Fig. 6. K_i was determined using the ^{125}I-sauvagine K_d value of 19 pM and the radioligand concentration (56–63 pM). The experiment involved measuring the ligand effect on ^{125}I-sauvagine binding in the presence of GTPγS (GTPγS-insensitive binding), and for these data the IC_{50} or EC_{50} value for inhibiting or enhancing ^{125}I-sauvagine binding is given. The ^{125}I-sauvagine bound as [ligand]→ ∞ values were calculated by dividing the specific binding asymptote for infinite ligand by that for no ligand (i.e., the right-hand asymptote by the left-hand asymptote of the curves as presented in Fig. 6). Data are mean ± S.E.M. (n = 3–5).

ligand	GTPγS-Sensitive			GTPγS-Insensitive		
	^{125}I-Sauvagine Bound as [ligand]→∞	−log K_i	K_i	^{125}I-Sauvagine Bound as [ligand]→∞	−log IC_{50}/EC_{50}	IC_{50}/EC_{50}
	%		*nM*	%		*nM*
CRF	0	9.83 ± 0.03	0.15	0	8.74 ± 0.23	1.8
NBI 30775	1 ± 1	8.80 ± 0.21	1.6	−10 ± 10	8.29 ± 0.06	5.2
NBI 34041	1 ± 1	8.37 ± 0.10	4.3	−6 ± 15	7.89 ± 0.10	13
NBI 35965	2 ± 1	8.66 ± 0.01	2.2	−2 ± 5	8.30 ± 0.18	5.5
NBI 38242	4 ± 1	7.02 ± 0.11	95	26 ± 1	6.39 ± 0.16	400
NBI 77173	14 ± 3	6.37 ± 0.04	430	220 ± 60	5.44 ± 0.12	3600
NBI 77165	−1 ± 16	7.39 ± 0.02	41	3200 ± 400	6.46 ± 0.07	340
NBI 77178	15 ± 2	6.61 ± 0.18	250	1700 ± 100	5.94 ± 0.05	1100
NBI 77172	20 ± 4	6.79 ± 0.06	160	65 ± 23	5.84 ± 0.06	1500

Scheme S1 is combined with the current low-resolution two-domain molecular model of CRF and nonpeptide ligand binding (Perrin et al., 1998; Nielsen et al., 2000; Hoare et al., 2004; Grace et al., 2007; Mesleh et al., 2007). CRF binds with moderate affinity to the ECD of the CRF_1 receptor. CRF then interacts with the transmembrane domain, an interaction that modulates receptor conformation, manifested by receptor activation and allosteric modulation by nonpeptide ligand. Specifically, CRF interaction with the transmembrane domain promotes the formation of the weakly active state R* and the fully activated state R**L, speculatively represented as a sequential pathway in Fig. 7. In this mechanism, NBI 35965 stabilizes the inactive state. CRF can bind the ECD, but CRF interaction with the transmembrane domain is weak at the inactive state, explaining the reduction of CRF affinity produced by NBI 35965. Because the level of the active state is reduced, NBI 35965 antagonizes receptor activation and signaling (mechanism 1, Fig. 7). By contrast, NBI 77173 promotes the weakly active state. CRF binds the ECD, and at the weakly active state, CRF interaction with the transmembrane domain is strengthened, explaining the increase of CRF affinity produced by NBI 77173. However, through enriching the weakly active state, NBI 77173 attenuates the transformation to the fully active state, resulting in antagonism of CRF-stimulated signaling (mechanism 2, Fig. 7). The presented mechanism represents our current understanding of the mechanism of receptor modulation by nonpeptide ligands, a mechanism that potentially provides a straightforward, intuitive, and quantitative framework to evaluate the receptor mechanism of other nonpeptide ligands. However, we cannot rule out the possibility that a different mechanism could also explain the experimental findings.

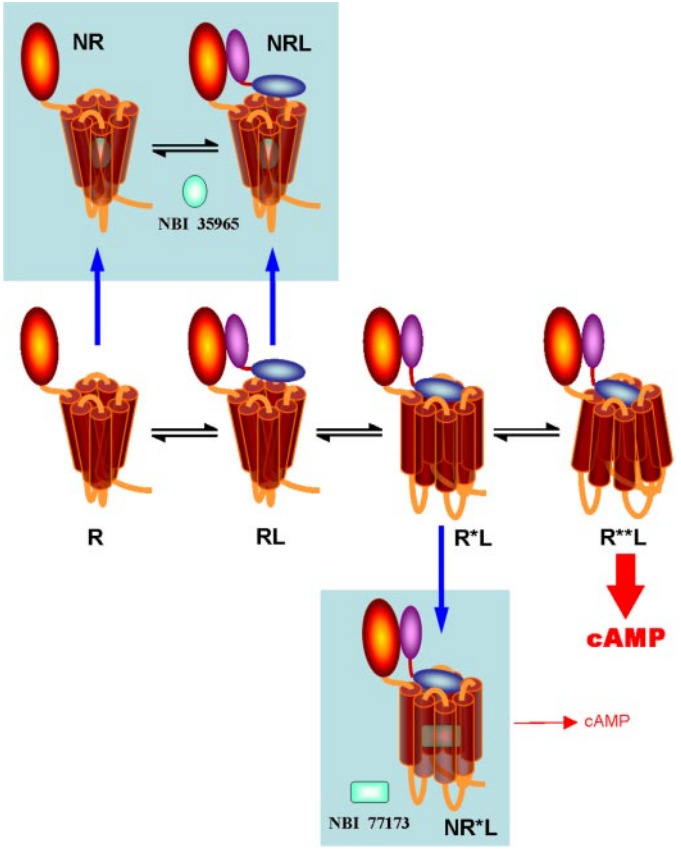

Fig. 7. Two-domain receptor state model of nonpeptide antagonism of the CRF_1 receptor. CRF binds to the CRF_1 receptor according to a two-domain model (Perrin et al., 1998; Nielsen et al., 2000; Hoare et al., 2004; Grace et al., 2007; Mesleh et al., 2007). Interaction with the extracellular domain provides an "affinity-trap" that facilitates binding to the membrane-spanning domain, which activates the receptor. Receptor activation by CRF involves conformational states of the receptor as illustrated in Supplemental Scheme S1: the inactive state (R), the weakly active state (R*) and the fully active state bound by CRF (R**L). Transition through these states is represented sequentially. Nonpeptide ligands antagonize CRF-stimulated cAMP signaling by two mechanisms. In mechanism 1, nonpeptide ligand (e.g., NBI 35965) stabilizes the inactive receptor state, inhibiting transition to the activated states. In mechanism 2, nonpeptide ligand (e.g., NBI 77173) stabilizes the weakly active state, inhibiting transition to the fully active state. The weakly active state does not signal detectably for the receptor expressed endogenously, resulting in antagonism of signaling. Other states in the full model (Supplemental Scheme S1) have been omitted for clarity.

The findings of this study are also potentially relevant to other receptor systems. The graded intrinsic activity of nonpeptide ligands, from inverse agonism to partial agonism, validates the hypothesis that the transmembrane domain of class B GPCRs modulates signaling. This finding has also been demonstrated with small peptide fragments on the PTH1 receptor, with activity varying from inverse agonism to full agonism (Shimizu et al., 2005). The partial agonism of NBI 77173 indicates that small, low-molecular-weight ligands can activate a class B GPCR. This is strongly demonstrated by nonpeptide full agonists of the glucagon-like peptide-1 receptor (Knudsen et al., 2007). The very low constitutive activity of the CRF_1 receptor might be common to the class B GPCR family, for which there are few if any previous reports of constitutive activity via an unmodified receptor. With respect to allosteric modulators of GPCRs, conformational plasticity has also been invoked to explain the action of class C GPCR allosteric modulators (Parmentier et al., 2002). Allosteric modulators of the CB1 receptor, a class A GPCR, have been demonstrated to enhance agonist binding but antagonize agonist signaling (Price et al., 2005), potentially consistent with mechanism 2 described in this study for the CRF_1 receptor. With respect to GPCRs generally, the CRF_1 receptor might represent a good candidate for crystallization studies to further elaborate GPCR structure beyond the X-ray crystal structures of the class A GPCRs rhodopsin (Palczewski et al., 2000) and β_2-adrenergic receptor (Rasmussen et al., 2007). Obtaining diffraction-quality crystals is facilitated by conformational stability, a requirement that could be aided by the very low constitutive activity of the CRF_1 receptor and the ability to further stabilize the inactive conformation with a nonpeptide inverse agonist (Rasmussen et al., 2007). The very high levels of CRF_1 receptor expression in mammalian expression systems (up to 100 pmol/mg) could also be useful in obtaining sufficient yields of functional, authentically processed protein.

In summary, this study further elaborates the mechanism of CRF_1 receptor regulation by nonpeptide ligands, evolving the mechanism to account for receptor conformational states. Two distinct mechanisms of antagonism were identified, which are consistent with the stabilization of different conformational states, potentially allowing the design of nonpeptide ligands of more diverse chemical structure. These findings will be useful in the further development of nonpeptide ligands targeting the CRF_1 receptor as potential therapies for anxiety, depression, and other stress-related disorders.

Acknowledgments

Khamkeo Khongsaly and Rajesh Huntley are acknowledged for excellent technical contributions, and R. Scott Struthers is gratefully acknowledged for useful discussions.

References

Assil-Kishawi I and Abou-Samra AB (2002) Sauvagine crosslinks to the second extracellular loop of the corticotropin-releasing factor type 1 receptor. *J Biol Chem* **277:**32558–32561.

Bale TL and Vale WW (2004) CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu Rev Pharmacol Toxicol* **44:**525–557.

Bruns RF and Fergus JH (1990) Allosteric enhancement of adenosine A1 receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol Pharmacol* **38:**939–949.

Chen C, Wilcoxen KM, Huang CQ, Xie YF, McCarthy JR, Webb TR, Zhu YF, Saunders J, Liu XJ, Chen TK, et al. (2004) Design of 2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylaminopyrazolo[1 ,5-a]pyrimidine (NBI 30775/R121919) and structure–activity relationships of a series of potent and orally active corticotropin-releasing factor receptor antagonists. *J Med Chem* **47:**4787–4798.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Dautzenberg FM, Higelin J, Brauns O, Butscha B, and Hauger RL (2002) Five amino acids of the *Xenopus laevis* CRF (corticotropin-releasing factor) type 2 receptor mediate differential binding of CRF ligands in comparison with its human counterpart. *Mol Pharmacol* **61:**1132–1139.

Ghanouni P, Gryczynski Z, Steenhuis JJ, Lee TW, Farrens DL, Lakowicz JR, and Kobilka BK (2001) Functionally different agonists induce distinct conformations in the G protein coupling domain of the β2 adrenergic receptor. *J Biol Chem* **276:**24433–24436.

Grace CR, Perrin MH, Gulyas J, Digruccio MR, Cantle JP, Rivier JE, Vale WW, and Riek R (2007) Structure of the N-terminal domain of a type B1 G protein-coupled receptor in complex with a peptide ligand. *Proc Natl Acad Sci U S A* **104:**4858–4863.

Gross RS, Guo Z, Dyck B, Coon T, Huang CQ, Lowe RF, Marinkovic D, Moorjani M, Nelson J, Zamani-Kord S, et al. (2005) Design and synthesis of tricyclic corticotropin-releasing factor-1 antagonists. *J Med Chem* **48:**5780–5793.

Hall DA (2000) Modeling the functional effects of allosteric modulators at pharmacological receptors: an extension of the two-state model of receptor activation. *Mol Pharmacol* **58:**1412–1423.

Hoare SR, Brown BT, Santos MA, Malany S, Betz SF, and Grigoriadis DE (2006) Single amino acid residue determinants of non-peptide antagonist binding to the corticotropin-releasing factor 1 (CRF1) receptor. *Biochem Pharmacol* **72:**244–255.

Hoare SR, Sullivan SK, Ling N, Crowe PD, and Grigoriadis DE (2003) Mechanism of corticotropin-releasing factor type I receptor regulation by nonpeptide antagonists. *Mol Pharmacol* **63:**751–765.

Hoare SR, Sullivan SK, Schwarz DA, Ling N, Vale WW, Crowe PD, and Grigoriadis DE (2004) Ligand affinity for amino-terminal and juxtamembrane domains of the corticotropin releasing factor type I receptor: regulation by G-protein and nonpeptide antagonists. *Biochemistry* **43:**3996–4011.

Holsboer F (2001) Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy. *J Affect Disord* **62:**77–91.

Hubbell WL, Altenbach C, Hubbell CM, and Khorana HG (2003) Rhodopsin structure, dynamics, and activation: a perspective from crystallography, site-directed spin labeling, sulfhydryl reactivity, and disulfide cross-linking. *Adv Protein Chem* **63:**243–290.

Ising M, Zimmermann US, Kunzel HE, Uhr M, Foster AC, Learned-Coughlin SM, Holsboer F, and Grigoriadis DE (2007) High-affinity CRF_1 receptor antagonist NBI-34041: preclinical and clinical data suggest safety and efficacy in attenuating elevated stress response. *Neuropsychopharmacology* **32:**1941–1949.

Kehne J and De Lombaert S (2002) Non-peptidic CRF1 receptor antagonists for the treatment of anxiety, depression and stress disorders. *Curr Drug Target CNS Neurol Disord* **1:**467–493.

Kenakin T (2007) Collateral efficacy in new drug discovery. *Trends Pharmacol Sci* **28:**359–361.

Knudsen LB, Kiel D, Teng M, Behrens C, Bhumralkar D, Kodra JT, Holst JJ, Jeppesen CB, Johnson MD, de Jong JC, et al. (2007) Small-molecule agonists for the glucagon-like peptide 1 receptor. *Proc Natl Acad Sci U S A* **104:**937–942.

Kobilka BK and Deupi X (2007) Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol Sci* **28:**397–406.

Kraetke O, Holeran B, Berger H, Escher E, Bienert M, and Beyermann M (2005) Photoaffinity cross-linking of the corticotropin-releasing factor receptor type 1 with photoreactive urocortin analogues. *Biochemistry* **44:**15569–15577.

Liaw CW, Grigoriadis DE, Lorang MT, De Souza EB, and Maki RA (1997) Localization of agonist- and antagonist-binding domains of human corticotropin-releasing factor receptors. *Mol Endocrinol* **11:**2048–2053.

Litvin Y, PasMantier R, Fleischer N, and Erlichman J (1984) Hormonal activation of the cAMP-dependent protein kinases in AtT20 cells. Preferential activation of protein kinase I by corticotropin releasing factor, isoproterenol, and forskolin. *J Biol Chem* **259:**10296–10302.

Mesleh MF, Shirley WA, Heise CE, Ling N, Maki RA, and Laura RP (2007) NMR structural characterization of a minimal peptide antagonist bound to the extracellular domain of the corticotropin-releasing factor 1 receptor. *J Biol Chem* **282:**6338–6346.

Miranda A, Koerber SC, Gulyas J, Lahrichi SL, Craig AG, Corrigan A, Hagler A, Rivier C, Vale W, and Rivier J (1994) Conformationally restricted competitive antagonists of human/rat corticotropin-releasing factor. *J Med Chem* **37:**1450–1459.

Montecucchi PC and Henschen A (1981) Amino acid composition and sequence analysis of sauvagine, a new active peptide from the skin of *Phyllomedusa sauvagei*. *Int J Pept Protein Res* **18:**113–120.

Nielsen SM, Nielsen LZ, Hjorth SA, Perrin MH, and Vale WW (2000) Constitutive activation of tethered-peptide/corticotropin-releasing factor receptor chimeras. *Proc Natl Acad Sci U S A* **97:**10277–10281.

Nikolaev VO, Hoffmann C, Bunemann M, Lohse MJ, and Vilardaga JP (2006) Molecular basis of partial agonism at the neurotransmitter α2A-adrenergic receptor and Gi-protein heterotrimer. *J Biol Chem* **281:**24506–24511.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, et al. (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* **289:**739–745.

Parmentier ML, Prezeau L, Bockaert J, and Pin JP (2002) A model for the functioning of family 3 GPCRs. *Trends Pharmacol Sci* **23:**268–274.

Perrin MH, Sutton S, Bain DL, Berggren WT, and Vale WW (1998) The first extracellular domain of corticotropin releasing factor-R1 contains major binding determinants for urocortin and astressin. *Endocrinology* **139:**566–570.

Perry SJ, Junger S, Kohout TA, Hoare SR, Struthers RS, Grigoriadis DE, and Maki RA (2005) Distinct conformations of the corticotropin releasing factor type 1 receptor adopted following agonist and antagonist binding are differentially regulated. *J Biol Chem* **280:**11560–11568.

Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, Goodwin R, McLean A, McIntosh L, Goodwin G, Walker G, et al. (2005) Allosteric modulation of the cannabinoid CB1 receptor. *Mol Pharmacol* **68:**1484–1495.

Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, et al. (2007) Crystal structure of the human β2 adrenergic G-protein-coupled receptor. *Nature* **450:**383–387.

Shimizu N, Dean T, Tsang JC, Khatri A, Potts JT Jr, and Gardella TJ (2005) Novel parathyroid hormone (PTH) antagonists that bind to the juxtamembrane portion of the PTH/PTH-related protein receptor. *J Biol Chem* **280:**1797–1807.

Smagin GN, Heinrichs SC, and Dunn AJ (2001) The role of CRH in behavioral responses to stress. *Peptides* **22:**713–724.

St-Denis Y, Di Fabio R, Bernasconi G, Castiglioni E, Contini S, Donati D, Fazzolari E, Gentile G, Ghirlanda D, Marchionni C, et al. (2005) Substituted tetraazaacenaphthylenes as potent CRF1 receptor antagonists for the treatment of depression and anxiety. *Bioorg Med Chem Lett* **15:**3713–3716.

Swaminath G, Xiang Y, Lee TW, Steenhuis J, Parnot C, and Kobilka BK (2004) Sequential binding of agonists to the β2 adrenoceptor. Kinetic evidence for intermediate conformational states. *J Biol Chem* **279:**686–691.

Yamada Y, Mizutani K, Mizusawa Y, Hantani Y, Tanaka M, Tanaka Y, Tomimoto M, Sugawara M, Imai N, Yamada H, et al. (2004) New class of corticotropin-releasing factor (CRF) antagonists: small peptides having high binding affinity for CRF receptor. *J Med Chem* **47:**1075–1078.

Zhang G, Huang N, Li YW, Qi X, Marshall AP, Yan XX, Hill G, Rominger C, Prakash SR, Bakthavatchalam R, et al. (2003) Pharmacological characterization of a novel nonpeptide antagonist radioligand, (±)-*N*-[2-methyl-4-methoxyphenyl]-1-(1-(methoxymethyl) propyl)-6-methyl-1H-1,2,3-triazolo[4,5-*c*]pyridin-4-amine ([^{3}H]SN003) for corticotropin-releasing factor 1 receptors. *J Pharmacol Exp Ther* **305:**57–69.

Zobel AW, Nickel T, Kunzel HE, Ackl N, Sonntag A, Ising M, and Holsboer F (2000) Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. *J Psychiatr Res* **34:**171–181.

Address correspondence to: Dr. Sam R. J. Hoare, Emerging Technologies Biomedical Research and Innovative Discovery Group, Neurocrine Biosciences Inc., 12790 El Camino Real, San Diego, CA 92130. E-mail shoare@neurocrine.com