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

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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 (CRF1) 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 $E_{\text{max}}$ 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 CRF1 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 adrenocorticotropic from pituitary corticotropes, which in turn stimulates corticosteroid release from the adrenal medulla. CRF also acts on CRF$_2$ receptors in cerebral cortical and limbic nuclei regulating behavior (Smagin et al., 2001). Studies of the physiology and pathophysiology of CRF$_1$ receptors have focused on their role in the regulation of the hypothalamic-pituitary-adrenal axis (Bale and Vale, 2004). CRF is secreted from the hypothalamus and stimulates the release of adrenocorticotropic from pituitary corticotropes, which in turn stimulates corticosteroid release from the adrenal medulla. CRF also acts on CRF$_2$ receptors in cerebral cortical and limbic nuclei regulating behavior (Smagin et al., 2001). Studies of the physiology and pathophysiology of CRF$_1$ receptors have focused on their role in the regulation of the hypothalamic-pituitary-adrenal axis (Bale and Vale, 2004). CRF is secreted from the hypothalamus and stimulates the release of adrenocorticotropic from pituitary corticotropes, which in turn stimulates corticosteroid release from the adrenal medulla. CRF also acts on CRF$_2$ receptors in cerebral cortical and limbic nuclei regulating behavior (Smagin et al., 2001).
physiology of the CRF system in animal models and humans implicate antagonism of central CRF₁ 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 CRF₁ receptor, a class B GPCR (Kehe and De Lombaert, 2002). Prototypical compounds are efficacious in animal models of CRF- and environmentally induced stress responses (Holsboer, 2001; Smagin et al., 2001; Kehe 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 CRF₁ 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 adenyl 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 CRF₁ receptor remains largely unknown. Multiple conformational states of the CRF₁ 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 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 CRF₁ 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, [Tyr²]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. [125I]-[Tyr²]astressin was synthesized using the chloramine-T method and purified by HPLC (specific activity, 2200 Ci/mmol). [3H]-[Tyr²]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 77175 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. [3H]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 overexpress the human CRF₁ receptor ([125I]-astressin Bmax = 100 ± 20 pmol/mg). This cell line, referred to here as CHO-CRF₁, 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 50μg/ml streptomycin. For radio-ligand binding assays, a Flp-In-CHO cell line expressing the human CRF₁ receptor was used that has been extensively characterized in radioligand binding experiments, with a Bmax 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-CRF₁ and AtT20 cells plated 18 h before the assay into 96-well plates (polylysine) coated for AtT20 cells. In initial experiments on CHO-CRF₁ 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$_2$ cells were plated at a density of 5000 cells/well, and AT20 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$_2$PO$_4$, 8.1 mM Na$_2$HPO$_4$, 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 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 [H]$^3$HNI 35965 and DPBS supplemented with 0.01% Triton X-100 for 125I-astressin and 125I-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 125I-astressin binding to cell membranes in the presence of 30 μM GTP-γS 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 GTP-γS, 25 μl of 125I-astressin, 25 μl of CRF, 50 μl of nonpeptide ligand, and 75 μl of membranes. The concentration of 125I-astressin varied between 63 and 110 pM, and the amount of membrane used was 70 to 150 ng/well. Saturation analysis indicated 125I-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 [H]$^3$HNI 35965 binding. Binding to the G-protein-uncoupled state of the receptor was measured in cell membranes by including 30 μM GTP-γS. Binding of each concentration of nonpeptide ligand was measured in duplicate. The reagents were added in the following order and volume: 25 μl of GTP-γS, 50 μl of [H]$^3$HNI 35965, 50 μl of nonpeptide ligand, and 75 μl of membranes. The concentration of 125I-astressin 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 [H]$^3$HNI 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 125I-sauvagine binding was measured in the absence and presence of 30 μM GTP-γS. Binding for each condition was measured in duplicate. The reagents were added in the following order and volume: 25 μl of 125I-sauvagine, 25 μl of GTP-γS or buffer alone, 50 μl of nonpeptide ligand, and 100 μl of membranes. The concentration of 125I-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 GTP-γS ($K_d$ value of 19 pM, $B_{max}$ value of 1.8 pmol/mg) and a lower-affinity state insensitive to GTP-γS, for which the affinity and $B_{max}$ value could not be reliably determined because saturation could not be reached at the highest concentration tested (5 nM). To assess the effect of nonpeptide ligand on GTP-γS-sensitive 125I-sauvagine binding, specific binding in the presence of GTP-γS was subtracted from specific binding in the absence of GTP-γS 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:

$$[RL^*] + [NRL^*] = \frac{[R_{TOT}][L^*][K_1](1 + C_{L}[N]/K_N)}{1 + ([L^*][K_1](1 + C_{L}[N]/K_N)) + ([L][K_1](1 + C_{L}[N]/K_N) + ([N]/K_N)} + NSB$$

(1)

**TABLE 1**

<table>
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<th>Ligand</th>
<th>log $K_{nm,peptide}$</th>
<th>log $K_{nm,peptide}$</th>
<th>log $K_{peptide}$</th>
<th>$K_{nm,peptide}$</th>
<th>$K_{nm,peptide}$</th>
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<th>$K_{nm,peptide}$</th>
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<tr>
<td>NBI 30775</td>
<td>0.30 ± 0.02</td>
<td>0.50</td>
<td>-0.96 ± 0.07</td>
<td>0.11</td>
<td>7.41 ± 0.11</td>
<td>39</td>
<td>9.06 ± 0.05</td>
<td>0.87</td>
<td>8.60 ± 0.06</td>
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<tr>
<td>NBI 34041</td>
<td>0.42 ± 0.05</td>
<td>0.38</td>
<td>-0.76 ± 0.06</td>
<td>0.18</td>
<td>7.11 ± 0.07</td>
<td>77</td>
<td>8.68 ± 0.33</td>
<td>2.1</td>
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<td>NBI 35965</td>
<td>0.15 ± 0.03</td>
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<td>-0.70 ± 0.05</td>
<td>0.20</td>
<td>7.23 ± 0.08</td>
<td>59</td>
<td>8.72 ± 0.10</td>
<td>1.9</td>
<td>8.69 ± 0.04</td>
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<tr>
<td>NBI 38242</td>
<td>0.29 ± 0.03</td>
<td>0.52</td>
<td>-0.48 ± 0.06</td>
<td>0.33</td>
<td>6.98 ± 0.14</td>
<td>100</td>
<td>6.72 ± 0.10</td>
<td>190</td>
<td>7.22 ± 0.05</td>
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<tr>
<td>NBI 77173</td>
<td>0.24 ± 0.04</td>
<td>0.53</td>
<td>0.96 ± 0.04</td>
<td>9.0</td>
<td>7.26 ± 0.07</td>
<td>53</td>
<td>8.10 ± 0.11</td>
<td>800</td>
<td>7.55 ± 0.06</td>
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<tr>
<td>NBI 77165</td>
<td>0.27 ± 0.04</td>
<td>1.9</td>
<td>0.91 ± 0.10</td>
<td>8.2</td>
<td>6.64 ± 0.01</td>
<td>115</td>
<td>6.14 ± 0.04</td>
<td>720</td>
<td>6.57 ± 0.19</td>
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<td>NBI 77164</td>
<td>0.12 ± 0.07</td>
<td>1.3</td>
<td>1.49 ± 0.04</td>
<td>31</td>
<td>7.26 ± 0.07</td>
<td>55</td>
<td>5.66 ± 0.06</td>
<td>2,200</td>
<td>6.14 ± 0.02</td>
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<td>NBI 77172</td>
<td>-0.10 ± 0.01</td>
<td>0.79</td>
<td>0.08 ± 0.05</td>
<td>1.2</td>
<td>7.26 ± 0.14</td>
<td>55</td>
<td>N.D.*</td>
<td>7.08 ± 0.05</td>
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N.D., not determined.

* The cooperative effect of NBI 77172 on peptide binding was too small to reproducibly determine nonpeptide ligand affinity, so in the fitting procedure, $K_{nm,peptide}$ was fixed at the value measured by displacement of [H]$^3$HNI 35965 binding.
where $R$ is receptor, $L^*$ is peptide radioligand ($^{125}$I-astressin), $L$ is unlabeled peptide ligand (CRF), $N$ is nonpeptide allosteric modulator, $K_i$, $K_e$, and $K_a$ are the corresponding equilibrium dissociation constants, $C_{C}$ 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), $[R]_0$ 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 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 (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 GTP$\gamma$S 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. 1A) 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 active and inactive 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 ($E_{\text{max}}$) 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-
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 CRF₁ 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 (Cᵦᵦᵦᵦ 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 (Cᵦᵦᵦᵦᵦᵦᵦ) 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.
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 $E_{\text{max}}$, 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...
the signaling efficacy of the receptor back down to that of R*. 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 CRF1 receptor was pharmacologically isolated using 125I-sauvagine, an amphibian analog of CRF (Montecucchi and Henschen, 1981). This peptide shares critical amino acid sequence determinants with CRF that are responsible for CRF1 receptor binding and activation (Montecucchi and Henschen, 1981). Binding of 125I-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 125I-sauvagine binding to the CRF1 receptor in CHO-CRF1 cell membranes by 86% (Fig. 6). The effect of nonpeptide ligand on 125I-sauvagine binding was measured in the absence and presence of GTPγS. GTPγS-sensitive 125I-sauvagine binding was then calculated by subtracting the level of 125I-sauvagine binding in the presence of GTPγS from the level in the absence of the nucleotide (Fig. 6).

NBI 77173 fully inhibited GTPγS-sensitive 125I-sauvagine binding (Fig. 6C), in marked contrast to the enhancement of CRF binding observed on the uncoupled CRF1 receptor (Fig. 2). It was necessary to subtract the 125I-sauvagine binding increment in the presence of GTPγS to determine that NBI 77173 fully inhibited GTPγS-sensitive 125I-sauvagine binding; NBI 77173 slightly enhanced 125I-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 CRF1 receptor uncoupled from G-protein. NBI 35965 fully inhibited GTPγS-sensitive 125I-sauvagine binding to the CRF1 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 125I-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 125I-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 125I-sauvagine binding to the G-protein-coupled CRF1 receptor. This state of the receptor was pharmacologically isolated as the increment of 125I-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 125I-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 $E_{\text{max}}$ 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 $E_{\text{max}}$ 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 $\beta_2$- and $\alpha_2$-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 (Kehe 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

<table>
<thead>
<tr>
<th>Ligand</th>
<th>GTP-Sensitive</th>
<th>GTP-Insensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125I-Sauvagine Bound as [ligand] =</td>
<td>125I-Sauvagine Bound as [ligand] =</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>$K_i$ (nM)</td>
</tr>
<tr>
<td>CRF</td>
<td>0</td>
<td>9.83 ± 0.03</td>
</tr>
<tr>
<td>NBI 30775</td>
<td>1 ± 1</td>
<td>8.80 ± 0.21</td>
</tr>
<tr>
<td>NBI 34041</td>
<td>1 ± 1</td>
<td>8.37 ± 0.10</td>
</tr>
<tr>
<td>NBI 35965</td>
<td>2 ± 1</td>
<td>8.66 ± 0.01</td>
</tr>
<tr>
<td>NBI 38242</td>
<td>4 ± 1</td>
<td>7.02 ± 0.11</td>
</tr>
<tr>
<td>NBI 77173</td>
<td>14 ± 3</td>
<td>6.37 ± 0.04</td>
</tr>
<tr>
<td>NBI 77185</td>
<td>−1 ± 16</td>
<td>7.79 ± 0.02</td>
</tr>
<tr>
<td>NBI 77178</td>
<td>15 ± 2</td>
<td>6.61 ± 0.18</td>
</tr>
<tr>
<td>NBI 77172</td>
<td>20 ± 4</td>
<td>6.79 ± 0.06</td>
</tr>
</tbody>
</table>

The effect of nonpeptide ligand on GTP-S-sensitive and insensitive 125I-sauvagine binding to the CRF$_1$ receptor was assessed by measuring GTP-S-sensitive 125I-sauvagine binding, as described in Fig. 6. The $K_i$ was determined using the 125I-sauvagine $K_i$ value of 19 nM and the radioligand concentration (56–63 pM). The experiment involved measuring the ligand effect on 125I-sauvagine binding in the presence of GTP-S (GTP-S-insensitive binding), and for these data the $IC_{50}$ or $EC_{50}$ value for inhibiting or enhancing 125I-sauvagine binding is given. The 125I-sauvagine bound as [ligand] = $K_i$ values were calculated by dividing the specific binding asymptote for infinite ligand by that for no ligand (i.e., the right-hand asymptote of the curves as presented in Fig. 6). Data are mean ± S.E.M. (n = 3–5).
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 CRF₁ 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.

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 CRF₁ 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 CRF₁ receptor. With respect to GPCRs generally, the CRF₁ receptor might represent a good candidate for crystalization studies to further elaborate GPCR structure beyond the X-ray crystal structures of the class A GPCRs rhodopsin (Palczewski et al., 2000) and β₂-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 CRF₁ receptor and the ability to further stabilize the inactive conformation with a nonpeptide inverse agonist (Rasmussen et al., 2007). The very high levels of CRF₁ receptor expression in mammalian expression systems (up to 100 pmol/mg) could also be useful in obtaining sufficient yields of functional, authentically processed protein.

Fig. 7. Two-domain receptor state model of nonpeptide antagonism of the CRF₁ receptor. CRF binds to the CRF₁ 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 activated 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.
References


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