Mechanism of Corticotropin-Releasing Factor Type I Receptor Regulation by Nonpeptide Antagonists

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ABSTRACT

Mechanisms of nonpeptide ligand action at family B G protein-coupled receptors are largely unexplored. Here, we evaluated corticotropin-releasing factor 1 (CRF1) receptor regulation by nonpeptide antagonists. The antagonist mechanism was investigated at the G protein-coupled (RG) and uncoupled (R) states of the receptor in membranes from Ltk- cells expressing the cloned human CRF1 receptor. R was detected with the antagonist 125I-astressin with 30 μM guanosine 5′-O-(3-thiotriphosphate) present, and RG detected using 125I-sauvagine. At the R state, nonpeptide antagonists antalarmin, NBI 35914, NBI 35965, and DMP-696 only partially inhibited 125I-astressin binding (22–32% maximal inhibition). NBI 35965 accelerated 125I-astressin dissociation and only partially increased the IC50 value of unlabeled sauvagine, CRF, and urocortin for displacing 125I-astressin binding (by 4.0–7.1-fold). Reciprocal effects at the R state were demonstrated using [3H]NBI 35965: agonist peptides only partially inhibited binding (by 13–40%) and accelerated [3H]NBI 35965 dissociation. These data are quantitatively consistent with nonpeptide antagonist and peptide ligand binding spatially distinct sites, with mutual, weak negative cooperativity (allosteric inhibition) between their binding. At the RG state the compounds near fully inhibited 125I-sauvagine binding at low radioligand concentrations (79–94 pM). NBI 35965 did not completely inhibit 125I-sauvagine binding at high radioligand concentrations (82 ± 1%, 1.3–2.1 nM) and slowed dissociation of 125I-sauvagine and 125I-CRF. The antagonist effect at RG is consistent with either strong allosteric inhibition or competitive inhibition at one of the peptide agonist binding sites. These findings demonstrate a novel effect of R-G interaction on the inhibitory activity of nonpeptide antagonists: Although the compounds are weak inhibitors of peptide binding to the R state, they strongly inhibit peptide agonist binding to RG. Strong inhibition at RG explains the antagonist properties of the compounds.

Corticotropin-releasing factor (CRF) is the principle mediator of the hypothalamic-pituitary-adrenal axis in the body’s response to stress (Vale et al., 1981; Rivier and Vale, 1983). This 41 amino-acid peptide binds to and activates the CRF1 receptor (Chen et al., 1993), which belongs to family B of the G protein-coupled receptor (GPCR) superfamily. The CRF1 receptor is activated by peptides related in amino acid sequence to CRF, including urocortin I (UCN I) and the amphibian peptide sauvagine (Dautzenberg and Hauger, 2002). Physiological studies have strongly implicated alteration of the CRF system in anxiety and depression (Holsboer, 1999; Gilligan et al., 2000; Grigoriadis et al., 2001). These compounds are CRF1 receptor-selective, block CRF1 receptor signaling in vitro, and demonstrate in vivo efficacy for reducing stress-related modulators and behaviors in animal models of neuropsychiatric disorders (Holsboer, 1999; Gilligan et al., 2000; Grigoriadis et al., 2001).

Mechanisms of peptide-ligand interaction with CRF receptors have been extensively investigated (Perrin and Vale, 1999; Grigoriadis et al., 2001). The extreme C terminus of CRF is required for high-affinity binding (Vale et al., 1981), whereas the N-terminal region of CRF is required for receptor activation (Rivier et al., 1984; Nielsen et al., 2000). These findings have been used to develop a high-affinity peptide antagonist, astressin (cyclo(30–

ABBREVIATIONS: CRF, corticotropin-releasing factor; GPCR, G protein-coupled receptor; UCN I, urocortin I; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; DPBS, Dulbecco’s phosphate-buffered saline; GTPγS, guanosine 5′-O-(3-thiotriphosphate); ANOVA, analysis of variance; R, G protein-uncoupled receptor state; RG, G protein-coupled receptor state; CP-154,526, butyl-[2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]ethylamine; NBI 27914, 5-chloro-N-(cyclopropylmethyl)-2-methyl-N-propyl-N’-(2,4,6-trichloropheny)-4,6-pyrimidinediamine hydrochloride; DMP-696, 4-(1,3-dimethoxyprop-2-ylamino)-2,7-dimethyl-8-(2,4-dichlorophenyl)pyrazolo[1,5-a]-1,3,5-triazine; SC241, [3-(2-bromo-4-isopropyl-phenyl)-5-methyl-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7-yl]-bis-(2-methoxy-ethyl)amine; R121919, 4-(1,3-dimethoxyprop-2-ylamino)-2,7-dimethyl-8-(2,4-dichlorophenyl)pyrazolo[1,5-a]-1,3,5-triazine.

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receptor interactions of nonpeptide ligands for the CRF1 receptor (Grigoriadis et al., 1997a,b; Perrin et al., 1998; Sydow et al., 1999). Collectively, these results suggest that the N-terminal portion of the ligand binds the J-domain of the receptor (for activation), and the C-terminal ligand region binds the receptor's N-domain (for high-affinity binding).

In contrast to peptide ligands, little is known regarding the receptor interactions of nonpeptide ligands for the CRF1 receptor. Receptor mutation has suggested that NBI 27914 binds to a site at least partially distinct from the peptide ligand binding regions (Liaw et al., 1997a; Nielsen et al., 2000; Assil et al., 2001) and contribute to ligand binding affinity (Liaw et al., 1997a,b; Perrin et al., 1998; Sydow et al., 1999). Collectively, these results suggest that the N-terminal portion of the ligand binds the J-domain of the receptor (for activation), and the C-terminal ligand region binds the receptor's N-domain (for high-affinity binding).

Materials and Methods

Materials. The peptides rat/human CRF, rat UCN I, sauvagine, astressin, and [Tyr10]astressin were synthesized by solid phase methodology on a Beckman Coulter 990 peptide synthesizer (Fullerton, CA) using t-Boc-protected amino acids. The assembled peptide was deprotected with hydrogen fluoride. The crude peptide product was purified by preparative HPLC, and the purity of the final product was assessed by analytical HPLC and mass spectrometric analysis using an ion-spray source. The peptides were dissolved in 10 mM acetic acid/0.1% bovine serum albumin (BSA) at a concentration of 1 mM and stored in 10- to 20-μl aliquots at −80°C. Aliquots were used once and any remaining solution discarded. 125I-[Tyr10]sauvagine and 125I-[Tyr10]astressin were obtained from PerkinElmer Life Sciences (Boston, MA) (specific activity of 2200 Ci/mmol), 125I-[Tyr10]astressin was synthesized utilizing the chloramine T method and purified by HPLC. [125I]NBI 35965 was custom synthesized by American Radiolabeled Chemicals (St. Louis, MO) (specific activity 25 Ci/mmol). Low-binding 96-well plates (no. 3605) were from Corning (Palo Alto, CA). G418 (geneticin), Dulbecco's phosphate-buffered saline (DPBS), and cell culture supplies were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from HyClone Laboratories (Logan, UT).

Cell Culture. Ltk- cells stably transfected with the human CRF1 receptor (Grigoriadis et al., 1994) (termed L-CRF1) were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 200 μg/ml G418.

Isolation of Cell Membranes. L-CRF1 cells were grown in 500-cm² tissue culture plates until confluent. The medium was removed and the cell monolayer washed once with 50 ml of DPBS per plate. Cells were then dislodged by scraping in 50 ml of DPBS per plate. Cells were collected in 250-ml centrifuge tubes and then pelleted by centrifugation at 800 g for 10 min at 4°C in a Beckman Coulter GS-6R centrifuge. The cell pellet was then resuspended in assay buffer [DPBS (1.5 mM KH2PO4, 8.1 mM Na2HPO4, 2.7 mM KCl, and 138 mM NaCl) supplemented with 10 mM MgCl2, 2 mM ethylene glycol-bis[β-aminoethyl]-N,N,N',N'-tetraacetic acid, pH 7.4, with NaOH], using 3 ml of buffer/500-cm² plate of cells. Cell lysis was then performed using a pressure cell, applying N2 at a pressure of 900 psi for 30 min at 4°C. Unbroken cells and larger debris were removed by centrifugation at 1200 g for 10 min at 4°C in a Sorvall RC 5C centrifuge (SM24 rotor). The cell membrane supernatant was then centrifuged at 45,000 g (Sorvall RC 5C centrifuge, SM24 rotor) and the resulting membrane pellet homogenized in assay buffer using a Biospec Products (Bartlesville, OK) model 985-370 tissue homogenizer on setting 5 for 30 s on ice. Membrane protein concentration was determined using the Coomassie method (Pierce Chemical, Rockford, IL), using BSA as the standard. Membranes were stored at −80°C before use.

Radioligand Binding Assays. Equilibrium binding of unlabeled ligands was measured in duplicate by inhibition of radioligand binding. 125I-[Tyr10]sauvagine, 125I-CRF, 125I-astressin, or [3H]NBI 35965 to L-CRF1 cell membranes. Buffer (30 μl), 20 μl of unlabeled ligand, 50 μl of radioligand, and 100 μl of L-CRF1 cell membranes were sequentially added to low protein-binding 96-well plates (no. 3605; Corning, NY). The assay mixture was incubated for 120 min at 12°C with GTPγS (30 μM final concentration) was included, in the 30 μl of buffer, to measure ligand binding to the G protein-uncoupled state of the receptor. In some assays GTPγS and NBI 35965 were included, added sequentially in volumes of 10 and 20 μl, respectively. The concentration of radioligand used was approximately 90 PM or 2 nM for 125I-sauvagine, 200 PM for 125I-sauvagine in the presence of GTPγS, 90 PM for 125I-CRF, 60 PM for 125I-astressin, and 2.5 nM for [3H]NBI 35965. The amount of membrane used per well was 2 to 5 μg for the peptide radioligands and 10 μg for [3H]NBI 35965. Dilution series of unlabeled ligands were prepared in low protein-binding 96-well plates. The assay mixture was incubated for 2 h at 21°C, a time period long enough to allow radioligand binding to closely approach its equilibrium binding asymptote (determined from radioligand association experiments), t_{1/2} determined from the observed association rate constant of 21, 5, and 15 min for 125I-sauvagine, 125I-astressin, and [3H]NBI 35965, respectively. Bound and free radioligand were then separated by rapid filtration, using UniFilter GF/C filters on a UniFilter-96 vacuum manifold (PerkinElmer Life Sciences). GF/C filters were pretreated for 20 to 40 min with 0.1% polyethyleneimine in DPBS and then pretreated, immediately before harvesting, by filtration with 0.2 ml/well 1% BSA/0.01% Triton X-100 in DPBS. The filter was washed four times with 0.2 ml/well 0.01% Triton X-100 in DPBS and then...
by adding 25 total binding (without unlabeled peptide or test agent) was measured
concentrated stocks into the unlabeled ligand solution. In each ex-
90 pM for 125I-CRF, 60 pM for 125I-astressin, and 2.5 nM for [3H]NBI
binding 96-well plates: 25 same time as the unlabeled analog of the radioligand. For the equil-
agents for modulation of radioligand dissociation were added at the
96 min after initiation of the dissociation phase of the experiment. Test
mining the radioligand bound at various time points (in duplicate)
association. Dissociation of the radioligand was measured by deter-
unlabeled analog of the radioligand added, to prevent radioligand
sauvagine and 125I-astressin, and [3H]NBI 35965. The amount of radioactivity recovered after the 2-h
for NBI 35965). Nonspecific binding, as a percentage of total radio-
amount of radioactivity recovered was not affected by the presence of a high concentration (1
C, a large excess of the unlabeled
°
recovered was not affected by the presence of GTP
30
l of L-CRF 1 cell membranes. The concentra-
ments indicated that the RO state of the receptor was present
where [RL] is total radioligand bound at time
1(fast) is the dissociation rate constant of the faster
dissociating component, and
1(slow) is the dissociation rate constant of the slower
dissociating component. In these analyses [RL - o] and
were held constant. [RL - o] was determined from linear regression
the time course of total binding measured as a control in the
dissociation phase of the assay, as the extrapolated value at 0
min. NSB was determined from the same analysis of the time course
of nonspecific binding.

Statistical comparison of multiple means was performed using
single-factor ANOVA, followed by post hoc analysis using the New-
man-Keuls test if significant difference was determined by ANOVA.
Statistical comparison of two means was performed using Student’s
t test (two-tailed).

Results

The mechanism of receptor regulation by nonpeptide an-
tagons was investigated by measuring ligand binding to
the CRF1 receptor. In this study, we evaluated the binding
mechanism at the different conformational states of the
CRF1 receptor in Ltk− cell membranes. Ligand binding to the
CRF1 receptor is regulated by receptor-G protein interaction,
an almost universal characteristic of GPCRs. The uncoupled
receptor state (R) binds agonists with lower affinity and can be
measured using the antagonist 125I-astressin with 30 μM
GTP-γ-S present. The receptor bound to G protein (RG) occu-
pies a state with high affinity for agonists and can be mea-
sured using the agonist radioligand 125I-sauvagine. A third,
minor state of the CRF1 receptor was identified (named here as
RO), which is insensitive to GTP-γ-S but which binds agon-
ists with high affinity. 1 125I-Sauvagine saturation experi-
ments indicated that the RO state of the receptor was present in
the absence of GTP-γ-S. Neither the Bmax nor Ks of 125I-
sauvagine for the RO state was affected 30 μM GTP–γ-S,
whereas 125I-sauvagine binding to RG was rendered unde-
tectable by GTP-γ-S. 1 Ligand binding to this third state can be
measured using 125I-sauvagine with 30 μM GTP-γ-S present.
We measured the effect of nonpeptide antagonists on peptide
radioligand binding to these three states of the CRF1 recep-
tor and also measured the effect of peptide ligands on
[3H]NBI 35965 binding to the R state.

Modulation of Equilibrium Peptide Antagonist
Binding to the R State of the CRF1 Receptor by
Nonpeptide Antagonists. We first examined the regulation of

1 S. Hoare, S. Sullivan, A. Pahuja, N. Ling, P. Crowe, and D. Grigoriadis,
manuscript in preparation.
the R state of the CRF₁ receptor. Initially, the effect of nonpeptide antagonists on radiolabeled antagonist binding was evaluated, by measuring the effect of nonpeptide antagonists on equilibrium ¹²⁵I-astressin binding to L-CRF₁ membranes in the presence of 30 μM GTPγS. ¹²⁵I-Astressin binding was not affected by any concentration of GTPγS tested (31.6 pM–100 μM), and the Kᵢ value of unlabeled astressin was not significantly different for the R and RG states.¹ Antalarmin, NBI 27914, NBI 35965, and DMP-696 failed to completely inhibit specific ¹²⁵I-astressin binding to the R state (Fig. 1A). At saturating concentrations (defined as the lower plateau of the inhibition curve), the compounds inhibited 20 to 32% of ¹²⁵I-astressin binding (Fig. 1A; Table 1). All three antagonists displayed high affinity for inhibiting ¹²⁵I-astressin binding (1.1–8.6 nM; Table 1). The partial inhibition of radioligand binding is suggestive of an allosteric mode of inhibition: Binding of ¹²⁵I-astressin to receptor saturated with nonpeptide antagonist is consistent with at least partially distinct binding sites for the two ligands (at the R state). In the Appendix, the ¹²⁵I-astressin inhibition data are analyzed using a quantitative model of allosteric modulation, the allosteric ternary complex model (Stockton et al., 1983; Ehlert, 1988; Lazareno and Birdsall, 1995). The fitted parameter estimates are presented in Table 1.

### Modulation of Peptide Antagonist Dissociation from the R State of the CRF₁ Receptor by Nonpeptide Antagonist

Modulation of peptide antagonist binding to R was investigated further in ¹²⁵I-astressin dissociation experiments. NBI 35965 accelerated dissociation of ¹²⁵I-astressin from L-CRF₁ membranes (with 30 μM GTPγS present) in a concentration-dependent, saturating manner, consistent with allosteric modulation of ¹²⁵I-astressin binding (Fig. 1B). NBI 35965 reduced the t₁/₂ for ¹²⁵I-astressin dissociation with a pEC₅₀ value of 7.89 ± 0.33 (EC₅₀ = 13 nM; Fig. 1C), lower than the compound’s potency for displacing equilibrium ¹²⁵I-astressin binding to R (pKᵢ = 8.87, Kᵢ = 1.4 nM; Table 1). The compound produced a maximal reduction of t₁/₂ of 1.5 ± 0.1-fold (Fig. 1C). ¹²⁵I-Astressin dissociation was biphasic in the absence and presence of NBI 35965 (Fig. 1, legend). The mechanism underlying biphasic dissociation is unknown. The observation might be due to multiple points of contact between ¹²⁵I-astressin and the receptor.

**Fig. 1.** Modulation of ¹²⁵I-astressin binding to the R state of the CRF₁ receptor by NBI 35965. Binding of ¹²⁵I-astressin to L-CRF₁ cell membranes was measured as described under Materials and Methods, in the presence of 30 μM GTPγS. A, inhibition of equilibrium ¹²⁵I-astressin binding by antalarmin, NBI 27914, NBI 35965, and DMP-696. The curves are the best fits to the allosteric ternary complex model (eq. 1; Appendix). The mean of the fitted values to eq. 1 are in Table 1. Data were normalized as the percentage of specific binding in the absence of nonpeptide antagonist, with nonspecific binding defined as binding in the presence of 320 nM astressin. Data points are the mean ± range of duplicate determinations. Data are from representative experiments that were performed three or four times with similar results. B, modulation of ¹²⁵I-astressin dissociation by NBI 35965. Dissociation of ¹²⁵I-astressin from L-CRF₁ cell membranes, in the presence of 30 μM GTPγS, was measured as described under Materials and Methods, in the absence or presence of a range of concentrations of NBI 35965. The curves are fits to a biexponential decay function, which provided a significantly better fit than a monoexponential function in all cases (p < 0.05). In the absence of NBI 35965, the mean fitted dissociation parameters were P₇₀ = 79 ± 12%, k⁺₁ = 0.060 ± 0.030 min⁻¹, k⁻₁ = 0.0066 ± 0.0021 min⁻¹. Data points are mean ± range of duplicate measurements. Data are from a representative experiment that was performed three times with similar results. D, effect of NBI 35965 on the half-time (t₁/₂) of ¹²⁵I-astressin dissociation. The curve is the best fit to a four-parameter logistic equation. Data are the mean ± S.E.M. of values from three experiments.
Table 1

Binding of nonpeptide antagonists to R, RG, and RO states of the CRF1 receptor in L-CRF1 cell membranes

Displacement of radioligand binding to L-CRF1 cell membranes was measured as described under Materials and Methods. Inhibition of 125I-sauvagine binding provides a measure of ligand affinity for the RG state of the CRF1 receptor. Ligand affinity for the R state was measured by displacement of 125I-astressin or [3H]NBI 35965 binding in the presence of 30 µM GTPyS. A third state of the receptor was identified in L-CRF1 cell membranes, which bound agonists with high affinity in a GTPyS-insensitive manner (RO). Ligand affinity for RO was measured by displacement of 125I-sauvagine binding with 30 nM GTPyS present. pKi values were obtained by fitting the displacement data to a single affinity-state competition model, followed by conversion of IC50 to Kd values (Cheng and Prusoff, 1973). In all cases, a two affinity-state model did not significantly improve the goodness of fit (p > 0.05).

<table>
<thead>
<tr>
<th>Antagonist Ligand</th>
<th>% Maximal Displacement</th>
<th>pKi nM</th>
<th>% Maximal Displacement</th>
<th>pKi nM</th>
<th>% Maximal Displacement</th>
<th>pKi nM</th>
<th>% Maximal Displacement</th>
<th>pKi nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antalarmin</td>
<td>101 ± 1</td>
<td>6.17 ± 0.08</td>
<td>92 ± 3</td>
<td>9.22 ± 0.26</td>
<td>101 ± 2</td>
<td>8.07 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBI 27914</td>
<td>100 ± 1</td>
<td>8.70 ± 0.18</td>
<td>113 ± 13</td>
<td>8.68 ± 0.66</td>
<td>99 ± 2</td>
<td>8.95 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBI 35965</td>
<td>99 ± 1</td>
<td>8.33 ± 0.10</td>
<td>94 ± 6</td>
<td>8.74 ± 0.06</td>
<td>100</td>
<td>8.87 ± 0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMP 696</td>
<td>101 ± 1</td>
<td>8.38 ± 0.08</td>
<td>110 ± 3</td>
<td>8.71 ± 0.07</td>
<td>98 ± 2</td>
<td>8.89 ± 0.22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The affinity of nonpeptide antagonists was determined using a model which assumes allosteric inhibition of 125I-astressin binding (the allosteric ternary model, eq. 1; see Appendix, pKi is equivalent to pKi in eq. 1). This analysis estimates nonpeptide affinity, and the cooperativity between nonpeptide and 125I-astressin binding (a). Data are the mean ± S.E.M. (n = 3–4).

Fig. 2. Effect of NBI 35965 on peptide agonist binding to the R state of the CRF1 receptor. The effect of NBI 35965 on binding of unlabeled peptide agonists to the R state was evaluated by measuring inhibition of 125I-astressin binding to membranes treated with equimolar concentrations of NBI 35965 and unlabeled CRF agonists. The antagonistic effect of NBI 35965 was measured by log (IC50) and expressed as a percentage of specific 125I-astressin binding. Data points are the mean ± range of duplicate determinations. 100 µM NBI 35965 did not detectably affect nonspecific binding (data not shown). Data points are from a representative experiment performed three times with similar results.
the presence of nonpeptide antagonists enabled us to examine modulation of unlabelled agonist binding to the R state. (Agonist binding to R was too weak to measure directly using agonist radioligands; Fig. 2.) Modulation of agonist binding was measured by inhibition of 125I-astressin binding by peptide agonist with GTPγS present, in the absence and presence of a range of concentrations of NBI 35965 (Fig. 2). NBI 35965 produced a rightward shift of the sauvagine, CRF, and UCN I inhibition curve (Fig. 2), indicating inhibition of agonist binding to the R state. However, incremental increases of NBI 35965 did not produce incremental increases of agonist IC50, as predicted by a competitive interaction between the two ligands. Rather, the extent of increase of agonist IC50 seemed to approach a limiting value (Fig. 2). In consequence, the fold-shift of agonist IC50 at 100 nM NBI 35965 (4.0 ± 0.8, 6.5 ± 2.2 and 7.1 ± 0.8 for sauvagine, CRF and UCN I, respectively) was much less than the fold-increase of agonist affinity predicted by competitive inhibition (57-fold, calculated using the Cheng-Prusoff equation; Cheng and Prusoff, 1973), assuming the affinity of NBI 35965 for the R state is 1.8 nM (Table 1). These observations are consistent with an allosteric interaction between NBI 35965 and agonist ligands at the R state: The limited increase of agonist IC50 suggests that binding of NBI 35965 only partially reduces the affinity of agonist binding to the receptor. In the Appendix, the allosteric ternary complex model has been used to quantify the allosteric effect, and the fitted parameters are provided in Table 2.

**Measurement of [3H]NBI 35965 Binding to the CRF1 Receptor.** In the experiments mentioned above, binding of nonpeptide antagonists has been measured indirectly, by measuring effects of the unlabeled compound on peptide radioligand binding. Although these experiments provide an estimate of the compounds’ affinity for the CRF1 receptor, they do not provide estimates of other important parameters of binding, such as Bmax and association and dissociation rate constants. In addition, radiolabeled nonpeptide antagonist binding would enable measurement of the effects of peptide ligands on nonpeptide binding, to further investigate the mechanism of action of the compounds. We therefore directly measured binding of [3H]NBI 35965 to the CRF1 receptor.

In saturation experiments [3H]NBI 35965 binding to L-CRF1 membranes was described by a single affinity-state model, with a pKd value of 9.25 ± 0.23 (n = 5, Kd = 0.56 nM; Fig. 3A). The number of sites labeled by [3H]NBI 35965 (6.1 ± 0.3pmol/mg) was similar to the number of sites labeled by the peptide antagonist 125I-astressin (7.7 ± 0.4 pmol/mg, n = 3). No specific [3H]NBI 35965 binding could be detected in membranes from Ltk- cells that were not transfected with the CRF1 receptor (data not shown). GTPγS did not appreciably affect equilibrium [3H]NBI 35965 binding (Fig. 4A; 5 ± 8% inhibition at 10 μM GTPγS). In addition, the affinity of unlabeled NBI 35965 was not significantly different for R and RG states (see below). Association and dissociation of [3H]NBI 35965 were both described by monoexponential processes, consistent with a single-affinity state of binding (Fig. 3, B and C). Steady-state binding, after equilibration, was reasonably stable for up to 3.5 h (Fig. 3C). Nonspecific binding did not change during the time course of [3H]NBI 35965 association and dissociation (Fig. 3, B and C). The association rate constant was 1.2 × 107 M −1 min −1 (n = 4). The lower limit of the [3H]NBI 35965 dissociation curve closely approached nonspecific binding (Fig. 3C), indicating reversible binding. Division of the dissociation rate constant (0.0087 ± 0.0020 min −1, n = 5, t1/2 = 80 min) by the association rate constant yielded a kinetically-derived Kd for [3H]NBI 35965 of 0.73 nM, in good agreement with the value measured by equilibrium binding (0.56 nM).

**Inhibition of [3H]NBI 35965 Binding to the R State of the CRF1 Receptor by Nonpeptide Antagonists.** The ability to directly label the nonpeptide antagonist binding site using [3H]NBI 35965 enabled us to test the hypothesis that antalarmin, NBI 27914, NBI 35965, and DMP-696 bind a common site on the CRF1 receptor. All the ligands fully inhibited [3H]NBI 35965 binding to L-CRF1 membranes (Table 1; measured in the presence of 30 μM GTPγS), consistent with either competitive or strong allosteric inhibition. NBI 27914 and DMP-696 at 1 μM concentration did not affect the dissociation rate of [3H]NBI 35965 (Fig. 5D), arguing against allosteric inhibition. These findings suggest that the nonpeptide antagonists bind the same site on the CRF1 receptor.

Full inhibition of [3H]NBI 35965 binding by the compounds enabled accurate measurement of their affinity for the R state. [The estimate from 125I-astressin inhibition assays was associated with a large standard error (Table 1), likely because of the weak maximal inhibition of 125I-astressin binding.] Antalarmin, NBI 27914, NBI 35965, and DMP-696 all

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**TABLE 2**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>pKd (1/REq)</th>
<th>α</th>
<th>pKd γ (1/REg)</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sauvagine</td>
<td>9.25 ± 0.14</td>
<td>0.33 ± 0.08b,c,d</td>
<td>6.36 ± 0.04</td>
<td>(430)</td>
</tr>
<tr>
<td>CRF</td>
<td>8.71 ± 0.07a</td>
<td>0.14 ± 0.03b</td>
<td>6.52 ± 0.03</td>
<td>(300)</td>
</tr>
<tr>
<td>UCN I</td>
<td>8.94 ± 0.17b</td>
<td>0.11 ± 0.03b</td>
<td>8.57 ± 0.07</td>
<td>(2.7)</td>
</tr>
<tr>
<td>Astrassine</td>
<td>8.87 ± 0.33a</td>
<td>0.65 ± 0.03c,d</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A., not applicable.

1 The pKd values of NBI 35965 for modulating binding of the four different ligands were not significantly different (p = 0.27, single-factor ANOVA). The α values for cooperativity between NBI 35965 and the four different ligands were significantly different (p = 0.0001). Post hoc analysis (Newman-Keuls test) was used to identify significant differences of α between pairs of peptide ligands.

2 Significantly different from the sauvagine α value.

3 Significantly different from the CRF α value.

4 Significantly different from the UCN I α value.

5 Equation 3 also provides estimates of unlabelled agonist affinity for the R state (pKd). To test the validity of the fit to eq. 3, these values were compared with those measured in the absence of a second, unlabeled ligand. The fitted value of agonist affinity (pKd) from eq. 3 (Fig. 2) was not significantly different from the Kd value obtained from competitive inhibition of 125I-astressin binding in the absence of NBI 35965 (p = 0.11, sauvagine pKd = 6.55 ± 0.7; p = 0.27; CRF pKd = 6.68 ± 0.09; p = 0.33; UCN I pKd = 8.72 ± 0.11; two-tailed Student’s t test, pKd values from manuscript in preparation). Equation 3 also provides estimates of cooperativity between NBI 35965 and 125I-astressin (β). The fitted value of β for analysis of NBI 35965 / agonist interaction was 0.75 ± 0.05, L ≥ 0.65, 0.65 ± 0.05 for sauvagine, CRF, and UCN I, respectively. These values were not significantly different from each other or from the value for NBI 35965 and 125I-astressin measured in the absence of unlabeled agonist (α from eq. 1, 0.65 ± 0.03; Fig. 1A) p = 0.40, single-factor ANOVA.)
bound with high affinity to the R state (0.6–2.1 nM; Fig. 4A; Table 1). The compounds bound with similar affinity in displacing [3H]NBI 35965 binding to the high-affinity state, although antalarmin showed a trend of higher affinity than the other compounds. In functional assays (inhibition of sau-
vagine-stimulated cAMP accumulation in whole cells) NBI 27914, NBI 35965, and DMP-696 were equivalently potent to each other (pIC50 values of 6.67 ± 0.13, 7.11 ± 0.12, and 7.31 ± 0.20, respectively, n = 6, 5, and 3, respectively). Antalarmin was slightly more potent (pIC50 of 7.79 ± 0.10, n = 3).

The Kᵢ value of unlabeled NBI 35965 (1.8 nM) was slightly higher (by 3.2-fold) than the Kᵢ value of [3H]NBI 35965. One possible explanation is a slight loss of unlabeled NBI 35965 during serial dilution in the displacement experiment, such that the actual concentration was less than that calculated by dilution. In contrast, the concentration of [3H]NBI 35965 in the saturation experiment was defined by radioactive counting of a sample of the radioligand dilution added to the assay.

**Fig. 4.** Inhibition of [3H]NBI 35965 binding to the R state of the CRF₁ receptor by unlabeled ligands. Inhibition of [3H]NBI 35965 binding to L-CRF₁ cell membranes was measured in the presence of 30 μM GTPγS, by nonpeptide antagonists (A) and by peptide ligands (B). The curves for CRF and UCN I are the best fit to the allosteric ternary complex model (eq. 2; see Appendix). Data were normalized as the percentage of specific binding in the absence of unlabeled ligand, with nonspecific binding defined as binding in the presence of 1 μM NBI 35965. Data points are the mean ± range of duplicate determinations.
Modulation of Equilibrium \(^{[3]H}\)NBI 35965 Binding to the R State of the CRF \(_1\) Receptor by Peptide Ligands.

The findings mentioned above suggest NBI 35965 allosterically regulates peptide agonist and antagonist binding to the R state of the CRF \(_1\) receptor. We examined the reciprocal effect of peptide ligands on NBI 35965 binding to the R state using \(^{[3]H}\)NBI 35965.

Agonist peptides sauvagine, CRF, and UCN I inhibited \(^{[3]H}\)NBI 35965 to L-CRF \(_1\) cell membranes with 30 \(\mu\)M GTP\(_S\) present (Fig. 4B). However, the peptide agonists only partially inhibited \(^{[3]H}\)NBI 35965 binding to the CRF \(_1\) receptor (Fig. 4B; Table 3). This finding suggests allosteric inhibition of \(^{[3]H}\)NBI 35965 binding to the R state by peptide agonists, because \(^{[3]H}\)NBI 35965 bound the CRF \(_1\) receptor saturated with these ligands. In the Appendix, the allosteric effect has been quantified using the allosteric ternary complex model, and the parameters are given in Table 3. The antagonist peptide astressin did not detectably inhibit \(^{[3]H}\)NBI 35965 binding (Fig. 4B; Table 3), suggesting that saturation of the receptor with astressin did not detectably affect the binding of \(^{[3]H}\)NBI 35965 under the conditions of the assay.

Modulation of \(^{[3]H}\)NBI 35965 Dissociation from the R State of the CRF \(_1\) Receptor by Peptide Ligands. Allosteric regulation of \(^{[3]H}\)NBI 35965 binding to the R state was further tested by measuring dissociation of the radioligand in the presence of GTP\(_S\). The agonists sauvagine, CRF, and UCN I accelerated dissociation of \(^{[3]H}\)NBI 35965 in a concentration-dependent and saturating manner (Fig. 5, A–C), consistent with allosteric modulation of \(^{[3]H}\)NBI 35965 binding. The effect was quantified by measuring the concentration dependence of the ligands for increasing the dissociation rate constant (\(k_{-1}\)) of \(^{[3]H}\)NBI 35965 (Fig. 5D). (Dissociation of \(^{[3]H}\)NBI 35965 was monophasic in the absence and presence of peptide ligands.) The pEC\(_{50}\) value for sauvagine, CRF, and UCN I was 6.24 ± 0.04, 6.38 ± 0.01, and 7.33 ± 0.02 respectively, with a corresponding maximal increase of the dissociation rate of 3.6 ± 0.5-, 5.3 ± 0.1-, and 7.0 ± 0.1-fold (\(n = 2\)). A saturating concentration of astressin (3.2 \(\mu\)M) did not significantly affect the dissociation rate of \(^{[3]H}\)NBI 35965 (Fig. 5D). This finding is in contrast to the modulation of \(^{125}\)I-astressin dissociation by NBI 35965 (Fig. 1). The reason for this difference is not presently clear.

Modulation of Equilibrium Peptide Agonist Binding to the RG State of the CRF \(_1\) Receptor by Nonpeptide Antagonists. Modulation of agonist binding to RG was first evaluated in equilibrium binding assays, by measuring inhibition of \(^{125}\)I-sauvagine binding to L-CRF \(_1\) cell membranes in the absence of GTP\(_S\). In these assays it was not possible to detect the RG state as a homogeneous population of binding sites, owing to the detection of the RO state by \(^{125}\)I-sauvagine.\(^1\) However, we were able to maximize the occupancy of RG relative to RO by using a low concentration of the radioligand (90 pM), because \(^{125}\)I-sauvagine binds with higher

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**Fig. 5.** Modulation of \(^{[3]H}\)NBI 35965 dissociation from the R state of the CRF \(_1\) receptor by peptide ligands. \(^{[3]H}\)NBI 35965 dissociation from L-CRF \(_1\) cell membranes was measured in the presence of 30 \(\mu\)M GTP\(_S\), in the presence of sauvagine (A), CRF (B), and UCN I (C). The curves are the best fit to a monoexponential dissociation equation. (In all cases, the biexponential equation did not improve the goodness of fit.) Data points are the mean ± range of duplicate determinations. Data are from representative experiments performed twice with similar results. The peptide diluent buffer (10 mM acetic acid/0.1% BSA, 25 \(\mu\)l in assay volume of 200 \(\mu\)l) did not affect the dissociation rate constant (0.0069 ± 0.0017 min\(^{-1}\) versus the control value of 0.0086 ± 0.0020 min\(^{-1}\)). D, effect of peptide and nonpeptide ligands on the dissociation rate constant of NBI 35965 (\(k_{-1}\)). The curves are the best fit to a four-parameter logistic equation. Data are the mean ± range of values from two experiments.
affinity to RG (43 pM) than to R (1.4 nM). Under these conditions the RG state represented 93% of the receptor-specific $[^{125}I]$-sauvagine binding (calculated from the dissociation constants above and $B_{max}$ values of 1.4 and 1.2 pmol/mg for RG and R, states, using a two independent affinity-state model.\(^1\)

A variety of nonpeptide antagonists (antalarmin, NBI 27914, NBI 35965, and DMP-696) fully inhibited $[^{125}I]$-sauvagine binding to L-CRF\(_1\) cell membranes, under conditions in which RG was the predominant state detected (Fig. 6; Table 1; 87–94 pm $[^{125}I]$-sauvagine). The compounds displayed high affinity for this effect (Table 1). Similarly, NBI 35965 near fully inhibited $[^{125}I]$-CRF binding with high affinity (96 ± 1% inhibition, pK\(_i\) = 8.44 ± 0.04, K\(_i\) = 3.7 nM; graphical data not shown).

The mechanism by which nonpeptide antagonists affect equilibrium agonist binding to RG was investigated using NBI 35965. We tested for the presence of deviation from competitive inhibition, by increasing the concentration of $[^{125}I]$-sauvagine in the inhibition assay. As described in the Appendix and in Stockton et al. (1983) and Ehler (1988), for an allosteric inhibitor the allosteric effect can become manifest as incomplete radioligand inhibition as the radioligand concentration is increased. When the $[^{125}I]$-sauvagine dose was increased to 1.3 to 2.1 nM (30–49-fold the K\(_i\) value of 43 pM), NBI 35965 incompletely inhibited radioligand binding (82 ± 1% inhibition; Fig. 6B), suggesting a more complex interaction than competitive inhibition.

**Modulation of Peptide Agonist Dissociation from the RG State of the CRF\(_1\) Receptor by Nonpeptide Antagonist.** Deviation from competitive inhibition of peptide binding to RG by NBI 35965 was tested further in radiolabeled agonist dissociation experiments. NBI 35965 slowed dissociation of $[^{125}I]$-sauvagine and $[^{125}I]$-CRF from L-CRF\(_1\) cell membranes in a concentration-dependent and saturating manner (Fig. 7, A and B). The slowing of radiolabeled agonist dissociation by NBI 35965 was in marked contrast to the effect of GTP\(_S\), which accelerated dissociation of $[^{125}I]$-sauvagine and $[^{125}I]$-CRF (Fig. 7, A and B).

The effect of NBI 35965 on radiolabeled agonist dissociation was quantified by measuring the half-time ($t_{1/2}$) of radiolabeled agonist dissociation in the presence of a range of NBI 35965 concentrations (Fig. 7C). The antagonist increased the dissociation $t_{1/2}$ of $[^{125}I]$-sauvagine and $[^{125}I]$-CRF with a pEC\(_{50}\) value of 6.87 ± 0.31 and 7.28 ± 0.27, respectively (n = 3; EC\(_{50}\) values of 130 and 52 nM, respectively). Therefore, higher concentrations of NBI 35965 are required

### Table 3

<table>
<thead>
<tr>
<th>Ligand</th>
<th>% Maximal Inhibition</th>
<th>pK(_i) (1/K(_i))</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sauvagine</td>
<td>13 ± 2</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CRF</td>
<td>29 ± 4</td>
<td>6.57 ± 0.05(^a)</td>
<td>0.16 ± 0.04(^c) (270)</td>
</tr>
<tr>
<td>UCN I</td>
<td>40 ± 7</td>
<td>8.81 ± 0.15(^b)</td>
<td>0.22 ± 0.02(^c) (1.6)</td>
</tr>
<tr>
<td>Astressin</td>
<td>1 ± 4</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

\(^a\) The affinity of agonists fitted by eq. 2 was not significantly different from the affinity measured by inhibition of $[^{125}I]$-astressin binding ($p = 0.58, pK\(_i\) = 6.68 ± 0.09$ for CRF; $p = 0.39, pK\(_i\) = 8.72 ± 0.11$ for UCN I; two-tailed Student’s t test; pK\(_i\) values from manuscript in preparation).

\(^b\) The value for the two ligands was not significantly different ($p = 0.45$; two-tailed Student’s t test). Data are the mean ± S.E.M. (n = 7 for sauvagine, n = 5 for CRF, n = 4 for UCN I, and n = 5 for astressin).

\(^c\) The values for the two ligands were not significantly different ($p = 0.22$; two-tailed Student’s t test)
to modulate dissociation of the agonist from RG (Fig. 7C) than to inhibit equilibrium binding of the agonist to RG (Fig. 6B). The maximum fold-increase of $t_{1/2}$ was 4.6 ± 1.5 and 2.2 ± 0.4 for $^{125}$I-sauvagine and $^{125}$I-CRF, respectively (Fig. 7C).

In the absence of NBI 35965, $^{125}$I-sauvagine and $^{125}$I-CRF dissociation was biphasic (Fig. 7, legend). Dissociation was also biphasic in the presence of all concentrations of NBI 35965 tested. The mechanism underlying biphasic agonist dissociation is unknown, but the observation may be related to the detection of a small amount of the Rₐ state as well as the RG state by $^{125}$I-sauvagine.

Modulation of Equilibrium Peptide Agonist Binding to the Rₒ State of the CRF₁ Receptor by Nonpeptide Antagonists. A minor fraction of the CRF₁ receptor population in L-CRF₁ cell membranes (16%) exists in a conformation that binds agonists with high affinity, but which is insensitive to GTPγS (termed Rₒ).¹ The pharmacological profile of nonpeptide antagonist activity at this state was measured by inhibition of $^{125}$I-sauvagine binding to L-CRF₁ cell membranes in the presence of 30 μM GTPγS. In this assay, antalarmin NBI 27914, NBI 35965, and DMP-696 fully inhibited binding of a low concentration of $^{125}$I-sauvagine (150–240 pM), displaying high affinity for this effect (Fig. 8; Table 1).

Comparison of Nonpeptide Antagonist Affinity for R, RG, and Rₒ States of the CRF₁ Receptor. The nonpeptide antagonist affinity for these three states of the CRF₁ receptor was compared using the $K_i$ value for inhibition of $[^3H]$NBI 35965 binding in the presence of GTPγS, $^{125}$I-sauvagine binding, and $^{125}$I-sauvagine in the presence of GTPγS, respectively. None of the antagonists appreciably discriminated between these states: the largest difference of affinity was only 3.3-fold (between RG and Rₒ for NBI 35965; Table 1). The nonpeptide antagonist affinity for R, RG, and Rₒ was not significantly different for antalarmin, NBI 27914, and DMP 696 ($p = 0.10, 0.09, and 0.12$, respectively; single-factor ANOVA). The affinity values were significantly different for NBI 35965 ($p = 0.0057$; single-factor ANOVA): the affinity for Rₒ (4.6 nM) was significantly different from the affinity for R (1.8 nM; $p < 0.01$) and RG (1.4 nM; $p < 0.01$, post hoc analysis using the Newman-Keuls test).

Discussion
Numerous nonpeptide antagonists have been developed for the CRF₁ receptor, as potential therapies for CRF-associated disorders such as anxiety and depression (Holsboer, 1999; Gilligan et al., 2000; Grigoriadis et al., 2001). However, little is known regarding their functional mechanism of action at

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**Fig. 7.** Modulation of radiolabeled agonist dissociation from the RG state of the CRF₁ receptor by NBI 35965. Dissociation of $^{125}$I-sauvagine (A) and $^{125}$I-CRF (B) from L-CRF₁ cell membranes was measured as described under Materials and Methods, in the absence of antagonist or in the presence of a range of concentrations of NBI 35965. The curves are fits to a biexponential decay function, which provided a significantly better fit than a monoeponential function in all cases ($p < 0.05$). In the absence of NBI 35965, the mean fitted parameters for $^{125}$I-sauvagine dissociation were $P_{(fast)} = 63 ± 5\%$, $k_{1(fast)} = 0.20 ± 0.09 \text{ min}^{-1}$, and $k_{2(fast)} = 0.0088 ± 0.0002 \text{ min}^{-1}$ and for $^{125}$I-CRF were $P_{(fast)} = 38 ± 3\%$, $k_{1(fast)} = 0.24 ± 0.07 \text{ min}^{-1}$, and $k_{2(fast)} = 0.0089 ± 0.0005 \text{ min}^{-1}$. Data points are mean ± range of duplicate measurements. Data are from representative experiments that were performed three times with similar results. C, effect of NBI 35965 on the half-time ($t_{1/2}$) of $^{125}$I-sauvagine and $^{125}$I-CRF dissociation. The curves are the best fit to a four-parameter logistic equation. Data are the mean ± S.E.M. of values from three experiments.
the receptor level. The aim of this study was to quantitatively evaluate the mechanism of action of four nonpeptide antagonists: antalarmin, NBI 27914, NBI 35965, and DMP-696. In addition, we compared the effects of these molecules at the G protein-coupled (RG) and uncoupled (R) states of the CRF1 receptor in Ltk- cell membranes. The principle findings are as follows: 1) At the R state, nonpeptide antagonists only partially inhibited peptide ligand binding and accelerated 125I-astressin dissociation. 2) Reciprocally, peptide agonists only partially inhibited [3H]NBI 35965 binding to the R state and accelerated [3H]NBI 35965 dissociation. 3) Antalarmin, NBI 27914, NBI 35965, and DMP-696 likely bind a common site on the receptor and modulate peptide ligand binding in a quantitatively similar manner. 4) Nonpeptide antagonists bind with similar affinity to the R and RG state. 5) At the RG state nonpeptide antagonists strongly inhibited peptide agonist binding (in marked contrast to their behavior at the R state), explaining their antagonist effect. 6) At the RG state deviations from simple competitive inhibition were detected. As described below, findings 1 and 2 for the R state support an allosteric mechanism by which nonpeptide antagonist and peptide ligand inhibit each other’s binding. Findings 5 and 6 for the RG state are consistent with either strong allosteric inhibition or competitive inhibition at one of the peptide agonist binding sites.

At the R state of the CRF1 receptor, four observations were consistent with an allosteric mechanism for nonpeptide antagonism, in which peptide and nonpeptide ligands bind to at least partially distinct sites (Appendix; Stockton et al., 1983; Ehlert, 1988; Lazareno and Birdsall, 1995): 1) Saturating concentrations of nonpeptide antagonists only partially inhibited equilibrium 125I-astressin binding and only partially reduced peptide agonist binding affinity. This suggests that peptide ligands can bind the receptor saturated with nonpeptide antagonist, consistent with at least partial spatial independence of their binding sites. 2) Reciprocally, saturating concentrations of peptide agonists only partially inhibited equilibrium [3H]NBI 35965 binding, suggesting that nonpeptide antagonist can bind the receptor saturated with peptide ligand. 3) Nonpeptide antagonist (NBI 35965) accelerated dissociation of 125I-astressin, consistent with nonpeptide antagonist binding the receptor-125I-astressin complex. (We were unable to measure the effect of NBI 35965 on peptide agonist dissociation from R, because binding of peptide agonist radioligands to R could not be detected.) 4) Peptide agonists accelerated [3H]NBI 35965 dissociation, suggesting peptide agonist binding to the receptor-[3H]NBI 35965 complex.

Other potential models were considered to explain these four findings for the R state. In the first model, nonpeptide antagonist binds to only a subpopulation of the receptor population bound by 125I-astressin. This model could explain partial inhibition of 125I-astressin binding by nonpeptide antagonists. However, a number of findings argue against this model. First, the model can only explain partial 125I-astressin inhibition if the receptor subpopulation that can bind nonpeptide antagonist is independent of the subpopulation that cannot (i.e., the populations do not interconvert). Under these conditions, NBI 35965 could not affect dissociation of 125I-astressin. Furthermore, the Bmax value of [3H]NBI 35965 (6.0 pmol/mg) was similar to that for 125I-astressin (7.7 pmol/mg), arguing against NBI 35965 selectively binding to a minor fraction of the receptor population. Finally, nonpeptide antagonists bound with similar affinity to the known different states of the receptor in L-CRF1 cell membranes (R, RG, and R0; Table 1). In the second potential model, two binding regions of the peptide ligand bind to two corresponding, spatially independent sites on the receptor (site 1 and site 2). This model is consistent with the known peptide binding mechanism (Perrin and Vale, 1999; Grigoriadis et al., 2001). In this model nonpeptide antagonist competitively inhibits peptide binding to the site 1, without affecting peptide binding to site 2. Examination of this model using simulated data indicates that it allows for partial inhibition of peptide binding by nonpeptide antagonist, partial inhibition of [3H]NBI 35965 binding by peptide ligand (provided that the peptide affinity for the site 1 is weak compared with site 2), and modulation of peptide ligand dissociation. However, the model does not allow modulation of [3H]NBI 35965 dissociation by peptide ligand. Therefore, of the models considered, only allosteric modulation fully accounts for the data obtained for the R state of the CRF1 receptor.

For other GPCRs, allosteric modulation is consistent with a theoretical model, the allosteric ternary complex model (Stockton et al., 1983; Ehlert, 1988; Lazareno and Birdsall, 1995; Trankle et al., 1999; Leppik and Birdsall, 2000). In this model, the behavior of the allosteric ligand (e.g., NBI 35965) is defined by its affinity for the receptor and by the cooperativity between binding of allosteric and orthosteric ligand (e.g., CRF). Data for the R state of the CRF1 receptor were fitted to the allosteric ternary complex model to quantify the allosteric effect. The analysis indicated negative cooperativity between NBI 35965 and peptide agonist binding. The negative cooperativity was weak; the greatest effect of NBI 35965 was on UCN I binding (α = 0.11, indicating that NBI 35965 binding reduces the affinity of UCN I by only 9-fold). Equilibrium binding and radioligand dissociation data are in good agreement with the model (Appendix), indicating that allosteric modulation is sufficient to account for the data for the R state. In particular, the data are fully consistent with

Fig. 8. Inhibition of 125I-sauvagine binding to the R0 state of the CRF1 receptor by nonpeptide antagonists. Inhibition of 125I-sauvagine binding to L-CRF1 cell membranes was measured in the presence of 30 μM GTPγS, as described under Materials and Methods. Under these conditions the radioligand binds an agonist high-affinity state of the CRF1 receptor, which is insensitive to GTPγS (R0). The curves are fits to a single-affinity state inhibition model. [A two-site model did not significantly improve the goodness of fit (p > 0.05).] Data were normalized as the percentage of specific binding in the absence of antagonist, with nonspecific binding defined as binding in the presence of 320 nM sauvagine. Data points are the mean ± range of duplicate determinations. Data are from representative experiments that were performed three times with similar results.
the reciprocity of the allosteric effect, that the cooperativity of NBI 35965 on peptide agonist binding is equal to the cooperativity of peptide agonist on [3H]NBI 35965 binding (Tables 2 and 3; Appendix). This reciprocal relationship has been demonstrated for gallamine and N-methylscopolamine at the M₂ muscarinic acetylcholine receptor (Trankle et al., 1999).

At the RG state, the effect of the nonpeptide antagonists on peptide agonist binding differed markedly from the R state. Nonpeptide antagonists antalarmin, NBI 27914, NBI 35965, and DMP-696 strongly inhibited agonist binding to RG, in contrast to their weak inhibition of binding to R. This finding demonstrates, for the first time, that the inhibitory action of a family B GPCR antagonist is dependent upon the conformational state of the receptor. The strong inhibition of peptide agonist binding to RG explains the antagonist properties of the compounds, because this state of the receptor is coupled, via subsequent G protein activation, to intracellular signaling pathways. At the RG state, deviations from competitive behavior were observed: NBI 35965 slowed radiolabeled agonist dissociation and incompletely inhibited [125I]-sauvagine binding at high radioligand concentrations. These observations can be explained by strong allosteric inhibition by the nonpeptide antagonist (Appendix) or by a model that assumes competitive inhibition at one of two peptide agonist-binding sites (see above). We could not distinguish these two models because it was not possible to unambiguously define [3H]NBI 35965 binding to the RG state, to determine whether peptide ligands affect [3H]NBI 35965 dissociation from RG (a necessary experiment to discriminate the models for the R state; see above).

In this study, we have evaluated the functional mechanism of nonpeptide antagonism of the CRF₁ receptor. The molecular mechanism underlying the effects requires further investigation. In our view, the data in this study are consistent with three plausible molecular mechanisms (Fig. 9). These mechanisms assume that peptide binds to the N- and J-domains (Perrin and Vale, 1999; Grigoriadis et al., 2001), that nonpeptide antagonist binds only the J-domain (Liaw et al., 1997a; Nielsen et al., 2000), and that an allosteric interaction is at least partially involved in the inhibition of peptide binding by nonpeptide antagonist (see above). In mechanism 1, nonpeptide antagonist binds to a site distinct from the peptide-binding site in the J-domain, and allosterically inhibits peptide binding to the J-domain (Fig. 9A). In mechanism 2, nonpeptide antagonist binding to the J-domain allosterically inhibits peptide binding to the N-domain (Fig. 9B). In mechanism 3, an extension of mechanism 2, nonpeptide antagonist binds to the same site in the J-domain as the peptide, competitively inhibiting peptide binding to the J-domain, whereas allosterically inhibiting peptide binding to the N-domain (Fig. 9C). Molecular biological approaches will be required to distinguish these models. The currently limited data are consistent with mechanism 1: mutation of His 199 (in transmembrane 3) to Val and Met276 (in transmembrane 5) to Ile increased the Kᵢ value of NBI 27914 for the CRF₁ receptor (40- and 200-fold, respectively), without affecting the binding affinity of CRF (Liaw et al., 1997a).

In summary, for the first time we have quantitatively evaluated the inhibitory mechanism of nonpeptide antagonists for the CRF₁ receptor. The allosteric ternary complex model was necessary and sufficient to account for the data for the R state. The compounds are weak allosteric inhibitors of peptide binding to the R state. In contrast, at the RG state nonpeptide antagonists strongly inhibited peptide agonist binding, demonstrating a previously unknown effect of R-G coupling on nonpeptide antagonist activity. The strong inhibitory activity at RG could be explained by either strong allosteric inhibition or competitive inhibition at one of the two peptide-binding sites. Strong inhibition of peptide binding to RG explains the antagonist activity of the compounds. These findings will be relevant to the further study and discovery of nonpeptide antagonists for the CRF₁ receptor, and potentially for other family B GPCRs.

**Appendix**

**Description of the Allosteric Ternary Complex Model.** Numerous observations in this study suggest an allosteric interaction between the binding of nonpeptide antagonists and peptide ligands to the CRF₁ receptor. (Allosteric modulation is defined here as the ability of ligand binding to one site to influence the binding of ligand to a second, at least partially distinct site on the receptor.) For other GPCRs allosteric modulation is well described by a simple model, the allosteric ternary complex model (Stockton et al., 1983; Ehler, 1988; Lazareno and Birdsell, 1995) shown in Scheme 1.

As derived previously (Lazareno and Birdsell, 1995), the equation describing the effect of N on the binding of L is as follows:

\[
[RL] + [NRL] = [R_{TOT}] + [L]K_L(1 + \alpha[N]K_N)K_N
\]

where \([R_{TOT}] \) is the total receptor concentration. The equation describing the effect of L on the binding of N is as follows:
In the experiments in Fig. 2, we tested the effect of NBI 35965 on unlabeled agonist binding, by inhibition of $^{125}$I-astressin binding by unlabeled agonist in the presence of a range of concentrations of NBI 35965. To analyze these data, the allosteric ternary complex model can be extended as follows to take into account the presence of the unlabeled agonist (L), assumed to bind the same site as $^{125}$I-astressin (L*) as shown in Scheme 2. The equation describing the binding of L* in the presence of both L and N is as follows:


(2)

Analysis of Cooperativity between Binding of Nonpeptide and Peptide Ligands at the R State of the CRF$_1$ Receptor using the Allosteric Ternary Complex Model.

In equilibrium binding assays, antalarmin, NBI 27914, NBI 35965, and DMP-696 inhibited $^{125}$I-astressin binding to the R state of the CRF$_1$ receptor (Fig. 1A), consistent with negative cooperativity. The data were fitted to eq. 1 using Prism 3.0, to analyze the data. For calculating the negative cooperativity for CRF or UCN I (0.11 and 0.14, respectively) was stronger than that for sauvagine (0.33; Table 2). However, in all cases the negative cooperativity at the R state of the CRF$_1$ receptor was weak; the lowest $\alpha$ value, 0.11 for NBI 35965 and UCN I, indicates that binding of NBI 35965 to the receptor reduced the UCN I binding affinity by only 9-fold.

As described above, the model predicts that the cooperative effect of N binding on the affinity of L for R is the same as the effect of L binding on the affinity of N for R (Trankle et al., 1999). This prediction was tested by measuring the effect of unlabeled peptides on equilibrium binding of $[^3H]$NBI 35965 to the R state (Fig. 4B). The parameters for the allosteric ternary complex model were estimated by fitting the data to eq. 2. The $\alpha$ values for CRF and UCN I versus $[^3H]$NBI 35965 binding (0.16 and 0.22, respectively; Table 3) were in good agreement with the $\alpha$ values for NBI 35965 versus CRF and UCN I binding (0.14 and 0.11, respectively; Fig. 2; Table 2).

In addition, the affinity of CRF and UCN I estimated from inhibition of $[^3H]$NBI 35965 binding (p$K_I$ values of 6.57 and 8.81; Fig. 4B; Table 3) were in good agreement with the p$K_I$ values obtained from inhibition of $^{125}$I-astressin binding to the R state (6.68 and 8.81). Unfortunately the inhibition of $[^3H]$NBI 35965 binding by astressin and sauvagine was too weak to allow reliable fitting of the data to eq. 2. The finding that astressin did not appreciably affect $[^3H]$NBI 35965 binding (Fig. 4B; Table 3) could be due to the high dose of $[^3H]$NBI 35965 used relative to its $K_a$ value (2.8–6.2-fold the $K_a$ of 0.6 nM). For negatively cooperative ligands, the extent of maximal radioligand inhibition is related to the concentration of radioligand; increasing the radioligand concentration relative to its $K_a$ value decreases the maximal inhibition of radioligand binding by allosteric ligand. As a result, the use of high $[^3H]$NBI 35965 concentrations could have prevented the detection of inhibition by astressin.

We next considered the allosteric interaction between NBI 35965 and peptide ligands in radioligand dissociation experiments, for the R state of the CRF$_1$ receptor. In the allosteric ternary complex model, binding of N can affect the dissociation of L from the receptor because N can bind the RL complex. Inhibition of the three concentrations of NBI 35965 were analyzed simultaneously using SigmaPlot 2000 (SPSS Science, Chicago, IL), with [L] and [N] as independent variables. NBI 35965 exerted negative cooperativity on the binding of all three peptide agonists (sauvagine, CRF, and UCN I; Table 2). This negative cooperativity was significantly stronger between NBI 35965 and peptide agonists ($\alpha$ values of 0.11–0.33) than between NBI 35965 and the peptide antagonist astressin ($\alpha$ = 0.65; Table 1). In addition, $\alpha$ differed significantly between the different agonists; negative cooperativity for CRF or UCN I (0.11 and 0.14, respectively) was stronger than that for sauvagine (0.33; Table 2). However, in all cases the negative cooperativity at the R state of the CRF$_1$ receptor was weak; the lowest $\alpha$ value, 0.11 for NBI 35965 and UCN I, indicates that binding of NBI 35965 to the receptor reduced the UCN I binding affinity by only 9-fold.

![Scheme 1](image1.png)

Scheme 1. Allosteric ligand (N) and orthosteric ligand (L) each bind independently to the receptor, defined by the equilibrium association constants $K_N$ and $K_L$, respectively. (Orthosteric ligand binds the endogenous ligand binding site on the receptor.) In addition, allosteric modulator can bind to the RL complex, forming the NRL ternary complex. Reciprocally, L can bind to NR forming NRL. The allosteric effect of N binding on the affinity of L for R is quantified by the cooperativity factor $\alpha$. (Due to thermodynamic conservation, the effect of N binding on the affinity of L for R is equal to the effect of L binding on the affinity of N for R.) Of relevance to this study, negative cooperativity ($\alpha < 1$) is manifest in equilibrium binding assays as a reduction of labeled L binding in the presence of N, and reciprocally a reduction of labeled N binding in the presence of L.

![Scheme 2](image2.png)

Scheme 2. $K_L$ is the equilibrium association constant of L* ($^{125}$I-astressin) binding to R, and $\beta$ is the cooperativity between L* and N binding to R.
plex (Lazareno and Birdsell, 1995). NBI 35965 accelerated 125I-astressin dissociation from the R state (Fig. 2B). In the dissociation assay NBI 35965 can only appreciably bind the RL complex. As a result, the concentration dependence of the allosteric effect reflects NBI 35965’s affinity for the RL complex. In principle, the affinity of N for the RL complex (pK_a) can be determined as the negative logarithm of the half-maximally effective concentration of N for changing the dissociation rate constant of L (Lazareno and Birdsell, 1995). This value can then be compared with the pK_a value measured by inhibition of equilibrium 125I-astressin binding (calculated from the fitted values of a and K_N; Table 2), to test the hypothesis that the same allosteric mechanism underlies both effects (Stockton et al., 1983). However, the affinity of N for RL can only be determined from the change of L’s k_−1 value if equilibrium between N and RL is rapidly established within the time frame of the dissociation phase of the assay (Lazareno and Birdsell, 1995). We did not attempt to determine pK_a from the 125I-astressin dissociation assay, because NBI 35965 associates slowly with the receptor (t_1/2 of 15 min for association of 2.5 nM[3H]NBI 35965, compared with a t_1/2 of 24 min for dissociation of 125I-astressin). In addition, this analysis can only be applied if the radioligand dissociates monophasically, whereas 125I-astressin dissociation was biphasic. These considerations notwithstanding, the pK_a value calculated from equilibrium binding (8.55; a_K_N = 2.8 nM) was within the effective concentration range of NBI 35965 for accelerating 125I-astressin dissociation (Fig. 1C). This finding is reasonably consistent with the hypothesis that the same mechanism underlies both the modulation of 125I-astressin dissociation and equilibrium 125I-astressin binding.

In the allosteric ternary complex model, L can affect dissociation of N because it can bind the NR complex. We tested the capacity of peptide ligands to modulate dissociation of [3H]NBI 35965 from the R state of the CRF_1 receptor (Fig. 5). Peptide agonists accelerated [3H]NBI 35965 dissociation from the R state. The pEC_50 value of sauvagine and CRF for increasing k_−1 of [3H]NBI 35965 was 6.24 and 6.38, respectively (Fig. 5D). The values for sauvagine and CRF probably provide reasonable estimates of the value of pK_a (see above), because it is likely that the high effective concentrations of peptide rapidly associated with the receptor, and dissociation of [3H]NBI 35965 was slow (t_1/2 of 80 min). The pEC_50 values for sauvagine and CRF are in reasonable agreement (within 1.5- and 3.2-fold, respectively) with the pK_a values calculated for modulation of equilibrium [3H]NBI 35965 binding (6.07 and 5.88, respectively). (The equilibrium pK_a value was calculated using the pK_a of inhibition of 125I-astressin binding (K, Table 2 for sauvagine; Table 3 for CRF).) The reasonable agreement between 1/αK_a from equilibrium and kinetic assays suggest that the same allosteric mechanism underlies regulation of equilibrium [3H]NBI 35965 binding and [3H]NBI 35965 dissociation. However, for CRF and sauvagine we could not determine whether the pEC_50 value for modulation of [3H]NBI 35965 dissociation better matched the equilibrium K_a value rather than the αK_a value, given the small degree of negative cooperativity between NBI 35965 and the peptides and the accumulated error in the equilibrium estimate of αK_a (from α and K_a). For UCN I, the concentration-response relationship for increasing [3H]NBI 35965’s k_−1 was steep (Fig. 5C; slope factor of 1.93) and the pEC_50 (7.33) was less than the pK_a value calculated for modulation of equilibrium [3H]NBI 35965 binding (7.97). One possible explanation for these observations is that association of lower concentrations of UCN I with the NR complex was rate-limiting, such that the effect of low concentrations on [3H]NBI 35965 dissociation was underestimated.

In summary, ligand binding data for the R state of the CRF_1 receptor are in good agreement with the allosteric ternary complex model. In particular, the negative cooperativity of NBI 35965 on peptide binding was very similar to negative cooperativity of peptide on [3H]NBI 35965 binding. This reciprocal modulation provides strong evidence for the allosteric ternary complex model (Tranckle et al., 1999). The data are reasonably consistent with the hypothesis that the same allosteric effect underlies modulation of equilibrium radioligand binding and modulation of radioligand dissociation. The allosteric ternary complex model is therefore sufficient to account for the data.

Analysis of NBI 35965 and Peptide-Ligand Interactions at the RG State of the CRF_1 Receptor Using the Allosteric Ternary Complex Model. The experimental findings for the RG state are consistent with allosteric modulation and/or competitive inhibition of one of the two peptide binding sites (see Discussion). Here, the data are analyzed using the allosteric ternary complex model, assuming that allosteric modulation is responsible for the experimental findings.

Inhibition of radiolabeled agonist binding indicates a substantially greater inhibitory effect of nonpeptide antagonists on peptide agonist binding to the RG state (Fig. 6), compared with the R state (Fig. 2). NBI 35965 near fully inhibited binding of low concentrations (87–94 pM) of 125I-sauvagine (99% inhibition) and 125I-CRF (96% inhibition). This finding is consistent with a competitive interaction between NBI 35965 and agonist peptides and/or a strong negatively cooperative interaction. We tested for negative cooperativity by increasing the radiolabeled agonist concentration in the inhibition assay by increasing the radiolabeled agonist concentration. As described above, the maximal extent of radioligand binding inhibition produced by an allosteric inhibitor is inversely proportional to the radioligand concentration. For a strong negatively cooperative interaction, the allosteric interaction can become manifest as incomplete radioligand inhibition as the radioligand dose is increased (Stockton et al., 1983; Ehler, 1988). When the 125I-sauvagine dose was increased to 1.3–2.1 nM (30–49-fold the K_a), NBI 35965 incompletely inhibited radioligand binding (Fig. 6B), suggestive of an allosteric interaction between NBI 35965 and 125I-sauvagine at the RG state. The maximal extent of inhibition was 82 ± 1%. These data for RG were analyzed using the allosteric ternary complex model (eq. 1; Fig. 6B), yielding an estimate of α of 0.0056 ± 0.0012, (pK_a = 9.15 ± 0.06), indicating much greater negative cooperativity than at the R state (α = 0.33; Table 2). The fitted mean parameters from the allosteric ternary complex model were then used to simulate a 125I-sauvagine versus NBI 35965 inhibition curve for the low concentration of 125I-sauvagine, to check that the data for this dose were compatible with the model. As shown in Fig. 6B (dashed line), the simulated curve is in reasonable agreement with the data for the low 125I-sauvagine concentration. Almost all binding is displaced, according to this
model, because the negative cooperativity is high, and the concentration of radioligand (relative to its $K_d$) is low.

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References


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