0026-895X/03/6303-751–765$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:751–765, 2003

Vol. 63, No. 3
2123/1049869
Printed in U.S.A.

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

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Received September 4, 2002; accepted December 12, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Mechanisms of nonpeptide ligand action at family B G protein-coupled receptors are largely unexplored. Here, we evaluated corticotropin-releasing factor 1 (CRF_1) 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 CRF_1 receptor. R was detected with the antagonist ^{125}I-astressin with 30 μM guanosine 5′-*O*-(3-thiotriphosphate present, and RG detected using ^{125}I-sauvagine. At the R state, nonpeptide antagonists antalarmin, NBI 27914, NBI 35965, and DMP-696 only partially inhibited ^{125}I-astressin binding (22–32% maximal inhibition). NBI 35965 accelerated ^{125}I-astressin dissociation and only partially increased the IC_{50} value of unlabeled sauvagine, CRF, and urocortin for displacing ^{125}I-astressin binding (by 4.0–7.1-fold). Reciprocal effects at the R state were demonstrated using [^{3}H]NBI 35965: agonist peptides only partially inhibited binding (by 13–40%) and accelerated [^{3}H]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 ^{125}I-sauvagine binding at low radioligand concentrations (79–94 pM). NBI 35965 did not completely inhibit ^{125}I-sauvagine binding at high radioligand concentrations (82 $\pm$ 1%, 1.3–2.1 nM) and slowed dissociation of ^{125}I-sauvagine and ^{125}I-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 CRF_1 receptor (Chen et al., 1993), which belongs to family B of the G protein-coupled receptor (GPCR) superfamily. The CRF_1 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). Based on these studies CRF_1 receptor antagonism has been proposed as a potential treatment for these conditions. Many nonpeptide antagonists of the CRF_1 receptor have been described, such as CP 154,526 (Chen et al., 1997), SC241 (Gilligan et al., 2000), NBI 27914 (Chen et al., 1996), antalarmin (Webster et al., 1996), DMP-696 (He et al., 2000), and R121919 (Grigoriadis et al., 2000). These compounds are CRF_1 receptor-selective, block CRF_1 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-dimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]ethylamine; NBI 27914, 5-chloro-*N*-(cyclopropylmethyl)-2-methyl-*N*-propyl-*N*′-(2,4,6-trichlorophenyl)-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.

33)[D-Phe^{12},Nle^{21,38},Glu^{30},Lys^{33}]CRF(12–41) (Miranda et al., 1994)]. CRF receptors are predicted to consist of a large extracellular N-terminal domain (N-domain), connected to the juxtamembrane region consisting of the transmembrane domains and intervening loops (J-domain) (Perrin and Vale, 1999; Grigoriadis et al., 2001). The N-domain is a determinant of high-affinity peptide ligand binding (Liaw et al., 1997b; Dautzenberg et al., 1998; Perrin et al., 1998; Wille et al., 1999; Assil et al., 2001; Hofmann et al., 2001; Perrin et al., 2001). Regions and residues in the J-domain are involved in receptor activation by peptide ligands (Sydow et al., 1999; 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).

In contrast to peptide ligands, little is known regarding the receptor interactions of nonpeptide ligands for the CRF_1 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). SC241 modulates peptide ligand dissociation and reduces E_{max} in adenylyl cyclase assays (Zaczek et al., 1997). Thus, some qualitative evidence suggests that nonpeptide ligands may act allosterically to inhibit peptide ligand binding to the CRF_1 receptor. (Allosterism 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.) However, little or no quantitative data exist to support this hypothesis.

In this study, we have comprehensively evaluated the functional mechanism by which nonpeptide ligands antagonize peptide ligand binding to the CRF_1 receptor. We have applied a quantitative model to ligand binding data to test the hypothesis that nonpeptide antagonists inhibit peptide ligand binding to the CRF_1 receptor via an allosteric mechanism. Moreover, the extent to which receptor-G protein interaction affects the nonpeptide antagonist mechanism is unknown. (The pharmacological behavior of GPCR ligands is frequently dependent upon the conformational state of the receptor; Kenakin, 2002). Here, the effect of receptor-G protein interaction has been investigated and shown to profoundly affect the inhibitory activity of the compounds. Finally, antagonist mechanisms have previously only been assessed indirectly using unlabeled compounds. In this study the use of [^{3}H]NBI 35965 enabled direct measurement of the antagonist's receptor binding kinetics and allowed us to validate the proposed allosteric mode of action of the compound.

Materials and Methods

Materials. The peptides rat/human CRF, rat UCN I, sauvagine, astressin, and [Tyr^0]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. ^{125}I-[Tyr^0]sauvagine and ^{125}I-[Tyr^0]ovine CRF were obtained from PerkinElmer Life Sciences (Boston, MA) (specific activity of 2200 Ci/mmol). ^{125}I-[Tyr^0]astressin was synthesized using the chloramine T method and purified by HPLC (specific activity 2200 Ci/mol). [^{3}H]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 CRF_1 receptor (Grigoriadis et al., 1994) (termed L-CRF_1) 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-CRF_1 cells were grown in 500-cm^2 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 KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 138 mM NaCl) supplemented with 10 mM $MgCl_2$, 2 mM ethylene glycol-bis[β-aminoethyl]-*N*,*N*,*N*′,*N*′-tetraacetic acid, pH 7.4, with NaOH], using 3 ml of buffer/500-cm^2 plate of cells. Cell lysis was then performed using a pressure cell, applying N_2 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 (^{125}I-sauvagine, ^{125}I-CRF, ^{125}I-astressin, or [^{3}H]NBI 35965) to L-CRF_1 cell membranes. Buffer (30 μl), 20 μl of unlabeled ligand, 50 μl of radioligand, and 100 μl of L-CRF_1 cell membranes were sequentially added to low protein-binding 96-well plates (no. 3605; Corning). In some assays guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS, 30 μM final concentration) was included, added 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 ^{125}I-sauvagine, 200 pM for ^{125}I-sauvagine in the presence of GTPγS, 90 pM for ^{125}I-CRF, 60 pM for ^{125}I-astressin, and 2.5 nM for [^{3}H]NBI 35965. The amount of membrane used per well was 2 to 5 μg for the peptide radioligands and 10 μg for [^{3}H]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 ^{125}I-sauvagine, ^{125}I-astressin, and [^{3}H]NBI 35965, respectively). Bound and free radioligand were then separated by rapid filtration, using UniFilter GF/C filters (PerkinElmer Life Sciences) on a UniFilter-96 vacuum manifold (PerkinElmer Life Sciences). GF/C filters were pretreated for 20 to 40 min with 0.1% polyethylenimine 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

dried under electric fans for 40 min to 1 h. After addition of scintillation fluid (40 μl/filter disc, Microscint 20; PerkinElmer Life Sciences), scintillation counts were measured in a Topcount NXT. The cpm resulting from emission of beta particles from ^{3}H and Auger electrons from ^{125}I were converted to dpm, using the predetermined counting efficiency of 30%. In all assays total radioligand bound to the filter (total binding) was less than 20% of the total amount of radioligand added (6–15% for ^{125}I-sauvagine, 2–3% for ^{125}I-sauvagine with 30 μM GTPγS present, 14–19% for ^{125}I-astressin, and 9–15% for [^{3}H]NBI 35965). Nonspecific binding was determined as the measured value in the presence of an excess of the unlabeled analog of the radioligand (320 nM for peptide radioligands and 1 μM for NBI 35965). Nonspecific binding, as a percentage of total radioligand added, was 0.7 to 1.0% for ^{125}I-sauvagine, 0.5 to 0.9% for ^{125}I-sauvagine with 30 μM GTPγS present, 2 to 4% for ^{125}I-astressin, and 2 to 4% for [^{3}H]NBI 35965. The total binding: nonspecific binding ratio was 6 to 17 for ^{125}I-sauvagine, 3 to 4 for ^{125}I-sauvagine with 30 μM GTPγS present, 5 to 11 for ^{125}I-astressin, and 3 to 6 for [^{3}H]NBI 35965. The amount of radioactivity recovered after the 2-h incubation was measured by withdrawing all the assay solution from the well and counting it. The amount recovered was >95% for ^{125}I-sauvagine and ^{125}I-astressin, and >85% for [^{3}H]NBI 35965, indicating minimal depletion of the radioligand concentration by nonspecific binding to the plate surface. The amount of radioactivity recovered was not affected by the presence of a high concentration (1 μM) of NBI 35965 or sauvagine. The total amount of radioligand added was measured by using a PerkinElmer Life Sciences Cobra II gamma counter for ^{125}I-labeled peptides (78% efficiency) and by using a PerkinElmer Life Sciences 1600TR liquid scintillation counter for [^{3}H]NBI 35965 (55% efficiency).

In [^{3}H]NBI 35965 saturation experiments the following were added sequentially to low protein-binding 96-well plates: 25 μl of buffer, 50 μl of radioligand, 25 μl of buffer or unlabeled ligand in buffer, and 100 μl of L-CRF_1 cell membranes. Nonspecific binding was measured by including 1 μM unlabeled NBI 35965. The assay mixture was incubated for 2 h at 21°C and then the cell membranes harvested and radioactivity counted as described above. Duplicate measurements were performed for each condition.

In radioligand dissociation assays, radioligand was first equilibrated with L-CRF_1 cell membranes and then a large excess of unlabeled analog of the radioligand added, to prevent radioligand association. Dissociation of the radioligand was measured by determining the radioligand bound at various time points (in duplicate) after initiation of the dissociation phase of the experiment. Test agents for modulation of radioligand dissociation were added at the same time as the unlabeled analog of the radioligand. For the equilibration phase, the following were added sequentially to low protein-binding 96-well plates: 25 μl of buffer or GTPγS in buffer, 50 μl of radioligand, and 100 μl of L-CRF_1 cell membranes. The concentration of radioligand used was approximately 90 pM for ^{125}I-sauvagine, 90 pM for ^{125}I-CRF, 60 pM for ^{125}I-astressin, and 2.5 nM for [^{3}H]NBI 35965. After a 2-h incubation at 21°C, a large excess of the unlabeled analog of the radioligand was added (in 25 μl, 320 nM final concentration for peptide ligands and 1 μM for NBI 35965). Test agents for modulation of radioligand dissociation were diluted from 40 times concentrated stocks into the unlabeled ligand solution. In each experiment, unlabeled ligand was added nearly simultaneously to each well, and all wells for an individual time point were harvested simultaneously. Nonspecific binding was measured by including the unlabeled analog in the equilibration phase of the experiment, and total binding (without unlabeled peptide or test agent) was measured by adding 25 μl of buffer at the initiation of the dissociation phase of the assay. Nonspecific binding and total binding was measured at each time point in the dissociation phase.

Data Analysis. Inhibition of radioligand binding was fitted to one-affinity state or two-affinity state competition models, and the best fit determined using a partial F-test, using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA). K_i was calculated using the method of Cheng and Prusoff (1973). Radioligand saturation data were fitted to one- and two-site saturation equations using Prism 3.0, and the best fit determined using a partial F-test. (In all cases, the one-site model provided the best fit to the data ($p > 0.05$).) Radioligand dissociation data were analyzed using the following monoexponential and biexponential decay functions, and the best fit determined using a partial F-test, using Prism 3.0:

$$[RL] = ([RL_{t=0}] - NSB)e^{-k_{-1} \cdot t} + NSB$$

$$[RL] = ([RL_{t=0}] - NSB)((P_{(fast)}/100)e^{-k_{-1(fast)}t} + (1 - (P_{(fast)}/100))e^{-k_{-1(slow)}t}) + NSB$$

where [RL] is total radioligand bound at time t, $[RL_{t=0}]$ is total radioligand bound at 0 min, k_{-1} the dissociation rate constant, and NSB is nonspecific radioligand bound. In the biexponential equation, $P_{(fast)}$ is the percentage of $[RL_{t=0}]$ that dissociates at the faster of the two rates, $k_{-1(fast)}$ is the dissociation rate constant of the faster dissociating component, and $k_{-1(slow)}$ is the dissociation rate constant of the slower dissociating component. In these analyses $[RL_{t=0}]$ and NSB were held constant. $[RL_{t=0}]$ was determined from linear regression of 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 Newman-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 antagonists was investigated by measuring ligand binding to the CRF_1 receptor. In this study, we evaluated the binding mechanism at the different conformational states of the CRF_1 receptor in Ltk^- cell membranes. Ligand binding to the CRF_1 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 ^{125}I-astressin with 30 μM GTPγS present. The receptor bound to G protein (RG) occupies a state with high affinity for agonists and can be measured using the agonist radioligand ^{125}I-sauvagine. A third, minor state of the CRF_1 receptor was identified (named here as R_O), which is insensitive to GTPγS but which binds agonist with high affinity.[1] ^{125}I-Sauvagine saturation experiments indicated that the R_O state of the receptor was present in the absence of GTPγS. Neither the B_{max} nor K_d of ^{125}I-sauvagine for the R_O state was affected 30 μM GTPγS, whereas ^{125}I-sauvagine binding to RG was rendered undetectable by GTPγS.[1] Ligand binding to this third state can be measured using ^{125}I-sauvagine with 30 μM GTPγS present. We measured the effect of nonpeptide antagonists on peptide radioligand binding to these three states of the CRF_1 receptor and also measured the effect of peptide ligands on [^{3}H]NBI 35965 binding to the R state.

Modulation of Equilibrium Peptide Antagonist Binding to the R State of the CRF_1 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 CRF_1 receptor. Initially, the effect of nonpeptide antagonists on radiolabeled antagonist binding was evaluated, by measuring the effect of nonpeptide antagonists on equilibrium ^{125}I-astressin binding to L-CRF_1 membranes in the presence of 30 μM GTPγS. ^{125}I-Astressin binding was not affected by any concentration of GTPγS tested (31.6 pM–100 μM), and the K_i 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 ^{125}I-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 ^{125}I-astressin binding (Fig. 1A; Table 1). All three antagonists displayed high affinity for inhibiting ^{125}I-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 ^{125}I-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 ^{125}I-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 CRF_1 Receptor by Nonpeptide Antagonist. Modulation of peptide antagonist binding to R was investigated further in ^{125}I-astressin dissociation experiments. NBI 35965 accelerated dissociation of ^{125}I-astressin from L-CRF_1 membranes (with 30 μM GTPγS present) in a concentration-dependent, saturating manner, consistent with allosteric modulation of ^{125}I-astressin binding (Fig. 1B). NBI 35965 reduced the $t_{1/2}$ for ^{125}I-astressin dissociation with a pEC_{50} value of 7.89 ± 0.33 (EC_{50} = 13 nM; Fig. 1C), lower than the compound's potency for displacing equilibrium ^{125}I-astressin binding to R (pK_i = 8.87, K_i = 1.4 nM; Table 1). The compound produced a maximal reduction of $t_{1/2}$ of 1.5 ± 0.1-fold (Fig. 1C). ^{125}I-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 ^{125}I-astressin and the receptor.

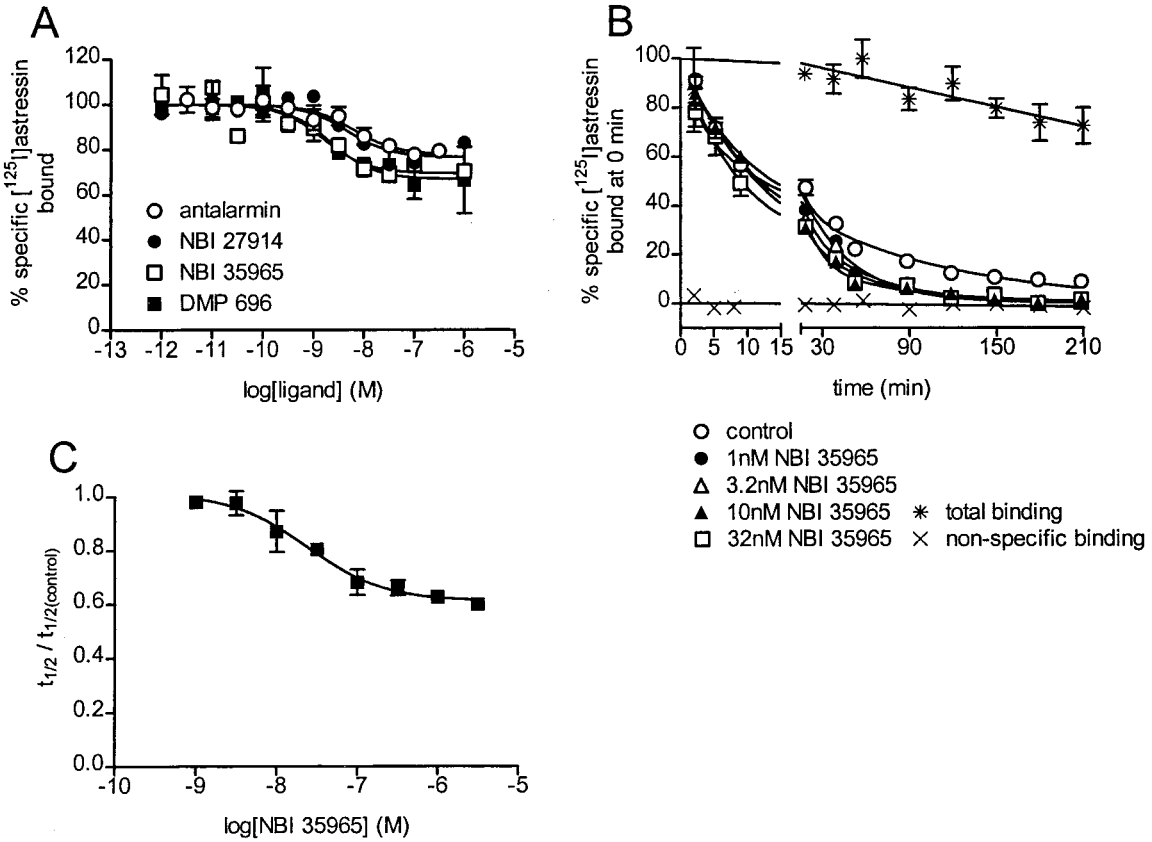


Fig. 1. Modulation of ^{125}I-astressin binding to the R state of the CRF_1 receptor by NBI 35965. Binding of ^{125}I-astressin to L-CRF_1 cell membranes was measured as described under *Materials and Methods*, in the presence of 30 μM GTPγS. A, inhibition of equilibrium ^{125}I-astressin binding by 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 ^{125}I-astressin dissociation by NBI 35965. Dissociation of ^{125}I-astressin from L-CRF_1 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 $P_{(fast)}$ = 79 ± 12%, $k_{-1(fast)}$ = 0.060 ± 0.030 min^{-1}, $k_{-1(slow)}$ = 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 ($t_{1/2}$) of ^{125}I-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 R_O states of the CRF1 receptor in L-CRF$_1$ cell membranes

Displacement of radioligand binding to L-CRF$_1$ cell membranes was measured as described under *Materials and Methods*. Inhibition of ^{125}I-sauvagine binding provides a measure of ligand affinity for the RG state of the CRF$_1$ receptor. Ligand affinity for the R state was measured by displacement of ^{125}I-astressin or [^{3}H]NBI 35965 binding in the presence of 30 μM GTPγS. A third state of the receptor was identified in L-CRF$_1$ cell membranes, which bound agonists with high affinity in a GTPγS-insensitive manner (R_O). Ligand affinity for R_O was measured by displacement of ^{125}I-sauvagine binding with 30 μM GTPγS present. pK_i values were obtained by fitting the displacement data to a single affinity-state competition model, followed by conversion of IC_{50} to K_i (Cheng and Prusoff, 1973). [In all cases, a two affinity-state model did not significantly improve the goodness of fit ($p > 0.05$)]. The K_d value used in the K_i calculation was 43 pM and 1.4 nM for ^{125}I-sauvagine at RG and R_O, respectively, 0.56 nM for [^{3}H]NBI 35965, and 70 pM for ^{125}I-astressin.

Antagonist Ligand	vs ^{125}I-Sauvagine (RG)		vs ^{125}I-Sauvagine + GTPγS (R_O)		vs [^{3}H]NBI 35965 + GTPγS (R)		vs ^{125}I-Astressin + GTPγS (R)		
	pK_i	% Maximal Displacement	pK_i	% Maximal Displacement	pK_i	% Maximal Displacement	pK_i	% Maximal Displacement	α
	nM		*nM*		*nM*		*nM*		
Antalarmin	9.42 ± 0.03 (0.38)	101 ± 1	8.71 ± 0.08 (2.0)	92 ± 3	9.22 ± 0.26 (0.61)	101 ± 2	8.07 ± 0.15[a] (8.6)	20 ± 1	0.63 ± 0.03[a]
NBI 27914	9.02 ± 0.06 (0.97)	100 ± 1	8.70 ± 0.18 (2.0)	113 ± 13	8.68 ± 0.06 (2.1)	99 ± 2	8.95 ± 0.06[a] (1.1)	28 ± 1	0.59 ± 0.01[a]
NBI 35965	8.84 ± 0.10 (1.4)	99 ± 1	8.33 ± 0.10 (4.6)	94 ± 6	8.74 ± 0.06 (1.8)	100	8.87 ± 0.33[a] (1.4)	22 ± 1	0.65 ± 0.03[a]
DMP-696	8.63 ± 0.11 (2.3)	101 ± 1	8.38 ± 0.08 (4.1)	110 ± 3	8.71 ± 0.07 (2.0)	98 ± 2	8.89 ± 0.22[a] (1.3)	32 ± 2	0.54 ± 0.02[a]

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

Modulation of Equilibrium Peptide Agonist Binding to the R State of the CRF$_1$ Receptor by Nonpeptide Antagonists. The measurable binding of ^{125}I-astressin in

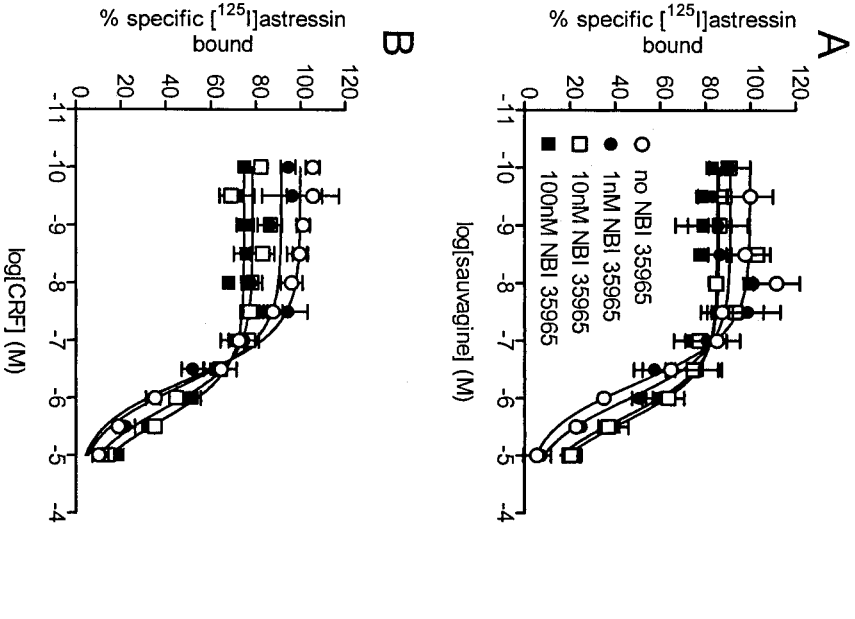


Fig. 2. Effect of NBI 35965 on peptide agonist binding to the R state of the CRF$_1$ receptor. The effect of NBI 35965 on binding of unlabeled peptide agonists to the R state was evaluated by measuring inhibition of ^{125}I-astressin binding by agonist in the presence of 30 μM GTPγS, in the absence and presence of NBI 35965. The unlabeled agonist peptides tested were sauvagine (A), CRF (B), and UCN I (C). Data were fitted to a model of allosteric modulation of peptide agonist binding by NBI 35965 (eq. 3). In this model, agonist and ^{125}I-astressin bind to a common site and NBI 35965 binds to a distinct site from which it allosterically regulates peptide agonist or ^{125}I-astressin binding. The curves are the best-fit curves to the data. Data were normalized as the percentage of specific binding in the absence of peptide agonist, with nonspecific binding defined as binding in the presence of 320 nM astressin. Note that NBI 35965 reduced the percentage specific binding in the absence of agonist, similar to the inhibition of ^{125}I-astressin by NBI 35965 in Fig. 1A. NBI 35965 did not detectably affect nonspecific binding (data not shown). Data points are the mean ± range of duplicate determinations. Data are from a representative experiment performed three times with similar results.

the presence of nonpeptide antagonists enabled us to examine modulation of unlabeled 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 ^{125}I-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 IC_{50}, as predicted by a competitive interaction between the two ligands. Rather, the extent of increase of agonist IC_{50} seemed to approach a limiting value (Fig. 2). In consequence, the fold-shift of agonist IC_{50} 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 IC_{50} 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.

TABLE 2

Estimates of the NBI 35965 affinity and cooperativity for allosteric modulation of peptide ligand binding to the R state of the CRF_1 receptor

The fitted parameters are those for the R state of the CRF_1 receptor in L-CRF_1 cell membranes. (30 μM GTPγS was included in the assays.) To determine the cooperativity (α) between the binding of NBI 35965 and astressin, data for inhibition of ^{125}I-astressin binding (Fig. 1A) were fitted to the allosteric ternary complex model (eq. 1, see Appendix). For the peptide agonists (sauvagine, CRF, and UCN I) K_N and α were estimated by fitting the data of Fig. 2 to the allosteric ternary complex model (eq. 3). Data are the mean ± S.E.M (n = 3). In all analyses the affinity constant of ^{125}I-astressin was fixed at the value measured in saturation experiments (1.43×10^{10} M^{-1}).

Peptide	pK_N ($1/K_N$)	α	pK_L ($1/K_L$)
	nM		*nM*
Sauvagine	9.25 ± 0.14[a] (0.56)	0.33 ± 0.08[b,c,d]	6.36 ± 0.04[e] (430)
CRF	8.71 ± 0.07[a] (1.9)	0.14 ± 0.03[b]	6.52 ± 0.03[f] (300)
UCN I	8.94 ± 0.17[a] (1.1)	0.11 ± 0.03[b]	8.57 ± 0.07[g] (2.7)
Astressin	8.87 ± 0.33[a] (1.4)	0.65 ± 0.03[c,d]	N.A.

N.A., not applicable.

[a] The pK_N 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 a between pairs of peptide ligands.

[b] Significantly different from the astressin α value.

[c] Significantly different from the CRF α value.

[d] Significantly different from the UCN I α value. Equation 3 also provides estimates of unlabeled agonist affinity for the R state (pK_L). 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 (pK_L) from eq. 3 (Fig. 2) was not significantly different from the K_i value obtained from competitive inhibition of ^{125}I-astressin binding in the absence of NBI 35965 ([e] p = 0.11, sauvagine pK_i = 6.55 ± 0.07; [f] p = 0.27, CRF pK_i = 6.68 ± 0.09; [g] p = 0.33, UCN I pK_i = 8.72 ± 0.11; two-tailed Student's t test, pK_i values from manuscript in preparation). Equation 3 also provides estimates of cooperativity between NBI 35965 and ^{125}I-astressin (β). The fitted value of β for analysis of NBI 35965 / agonist interaction was 0.75 ± 0.05, 0.73 ± 0.05, and 0.68 ± 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 ^{125}I-astressin measured in the absence of unlabeled agonist (α from eq. 1, 0.65 ± 0.03; Fig. 1A) (p = 0.40, single-factor ANOVA).

Measurement of [^{3}H]NBI 35965 Binding to the CRF_1 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 CRF_1 receptor, they do not provide estimates of other important parameters of binding, such as B_{max} 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 [^{3}H]NBI 35965 to the CRF_1 receptor.

In saturation experiments [^{3}H]NBI 35965 binding to L-CRF_1 membranes was described by a single affinity-state model, with a pK_d value of 9.25 ± 0.23 (n = 5, K_d = 0.56 nM; Fig. 3A). The number of sites labeled by [^{3}H]NBI 35965 (6.1 ± 0.3pmol/mg) was similar to the number of sites labeled by the peptide antagonist ^{125}I-astressin (7.7 ± 0.4 pmol/mg, n = 3).[1] No specific [^{3}H]NBI 35965 binding could be detected in membranes from Ltk^- cells that were not transfected with the CRF_1 receptor (data not shown). GTPγS did not appreciably affect equilibrium [^{3}H]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 [^{3}H]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 [^{3}H]NBI 35965 association and dissociation (Fig. 3, B and C). The association rate constant was $1.2 \times 10^7 \pm 0.7 \times 10^7$ M^{-1} min^{-1} (n = 4). The lower limit of the [^{3}H]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, $t_{1/2}$ = 80 min) by the association rate constant yielded a kinetically-derived K_d for [^{3}H]NBI 35965 of 0.73 nM, in good agreement with the value measured by equilibrium binding (0.56 nM).

Inhibition of [^{3}H]NBI 35965 Binding to the R State of the CRF_1 Receptor by Nonpeptide Antagonists. The ability to directly label the nonpeptide antagonist binding site using [^{3}H]NBI 35965 enabled us to test the hypothesis that antalarmin, NBI 27914, NBI 35965, and DMP-696 bind a common site on the CRF_1 receptor. All the ligands fully inhibited [^{3}H]NBI 35965 binding to the R state (Fig. 4A; 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 [^{3}H]NBI 35965 (Fig. 5D), arguing against allosteric inhibition. These findings suggest that the nonpeptide antagonists bind the same site on the CRF_1 receptor.

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

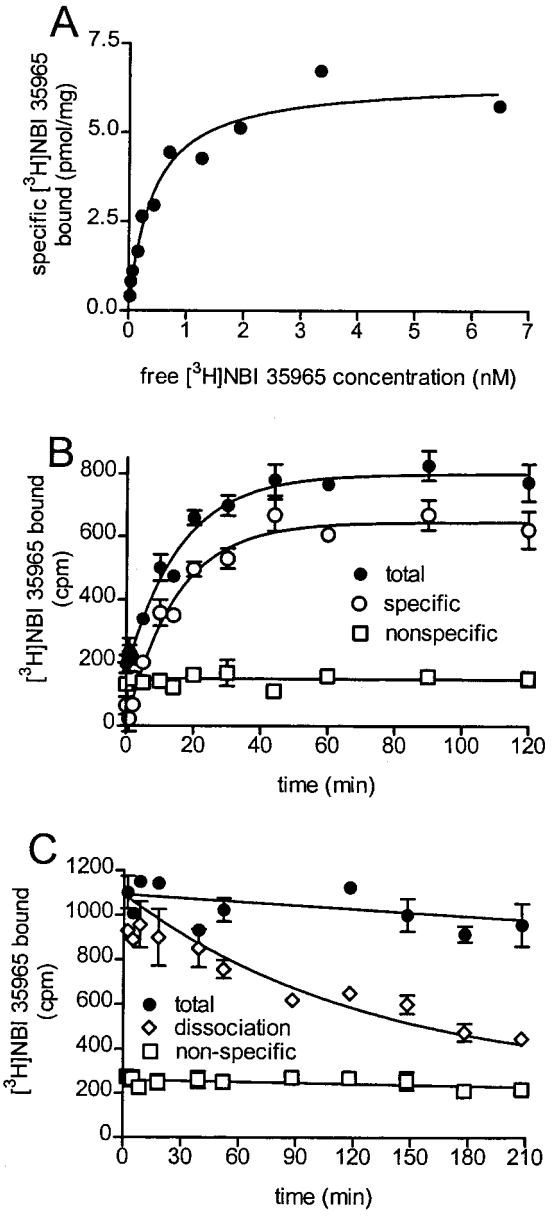


Fig. 3. Binding of [^{3}H]NBI 35965 to the CRF$_1$ receptor. A, [^{3}H]NBI 35965 saturation of L-CRF$_1$ cell membranes. The dependence of specific radioligand binding on the free radioligand concentration was fitted to a single affinity-state binding isotherm. [A two affinity-state model did not significantly improve the fit ($p > 0.05$).] Data are from a representative experiment, performed five times with similar results. The pK_d value for [^{3}H]NBI 35965 from the experiment shown was 9.39 (K_d of 0.41 nM). B, association time course of [^{3}H]NBI 35965 binding to L-CRF$_1$ cell membranes. The total concentration of [^{3}H]NBI 35965 added was 2.4 nM. The observed association rate constant was 0.0681 min^{-1}. The calculated association rate constant from this experiment was 2.5×10^7 M^{-1} min^{-1} (assuming a dissociation rate constant of 0.0087 min^{-1}; see text). Nonspecific binding was measured by including 1 μM NBI 35965, total binding measured in the absence of NBI 35965, and specific binding calculated by subtracting the former from the latter. Data are from a representative experiment performed four times with similar results. C, dissociation time course of [^{3}H]NBI 35965 binding to L-CRF$_1$ cell membranes. "Dissociation" binding was measured by adding NBI 35965 (1 μM final concentration) after equilibration of [^{3}H]NBI 35965 and the CRF1 receptor. "Total" binding was measured by adding the same volume of buffer after equilibration. "Nonspecific" binding measured by adding NBI 35965 (1 μM) before equilibration. Data are from a representative experiment performed five times with similar results. The curves are fits to monoexponential association and dissociation equations. The straight lines are linear regression fits. Data points are the mean ± range of duplicate measurements.

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 [^{3}H]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 sauvagine-stimulated cAMP accumulation in whole cells) NBI 27914, NBI 35965, and DMP-696 were equivalently potent to each other (pIC_{50} 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 (pIC_{50} of 7.79 ± 0.10, n = 3).

The K_i value of unlabeled NBI 35965 (1.8 nM) was slightly higher (by 3.2-fold) than the K_d value of [^{3}H]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 [^{3}H]NBI 35965 in the saturation experiment was defined by radioactive counting of a sample of the radioligand dilution added to the assay.

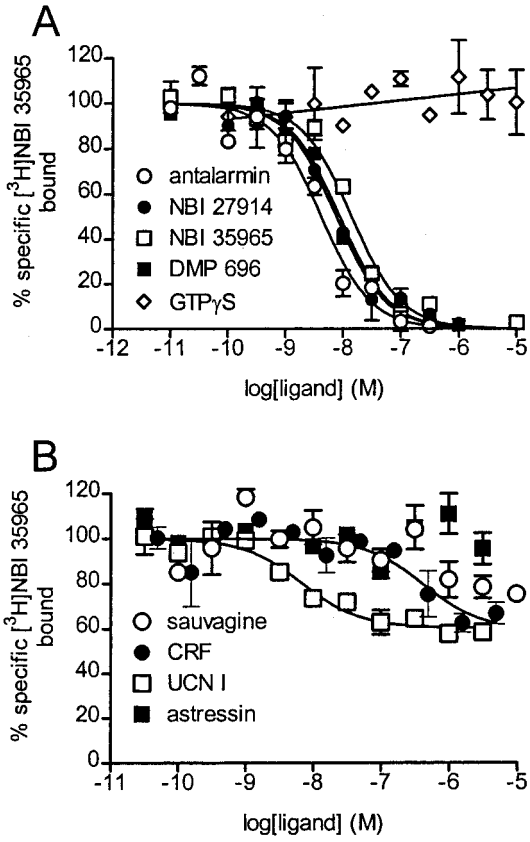


Fig. 4. Inhibition of [^{3}H]NBI 35965 binding to the R state of the CRF$_1$ receptor by unlabeled ligands. Inhibition of [^{3}H]NBI 35965 binding to L-CRF$_1$ cell membranes was measured in the presence of 30 μM GTPγS, by nonpeptide antagonists (A) and by peptide ligands (B). In addition, the effect of a range of concentrations of GTPγS on [^{3}H]NBI 35965 binding is also shown (A). The curves for nonpeptide antagonists are fits to a single-affinity state inhibition model. [A two-site model did not significantly improve the goodness of fit ($p > 0.05$).] 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. Data are from representative experiments, performed three to seven times with similar results.

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 μM GTPγS 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 GTPγS. 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 μM) 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 GTPγS. 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 R_O state by ^{125}I-sauvagine.[1] However, we were able to maximize the occupancy of RG relative to R_O by using a low concentration of the radioligand (90 pM), because ^{125}I-sauvagine binds with higher

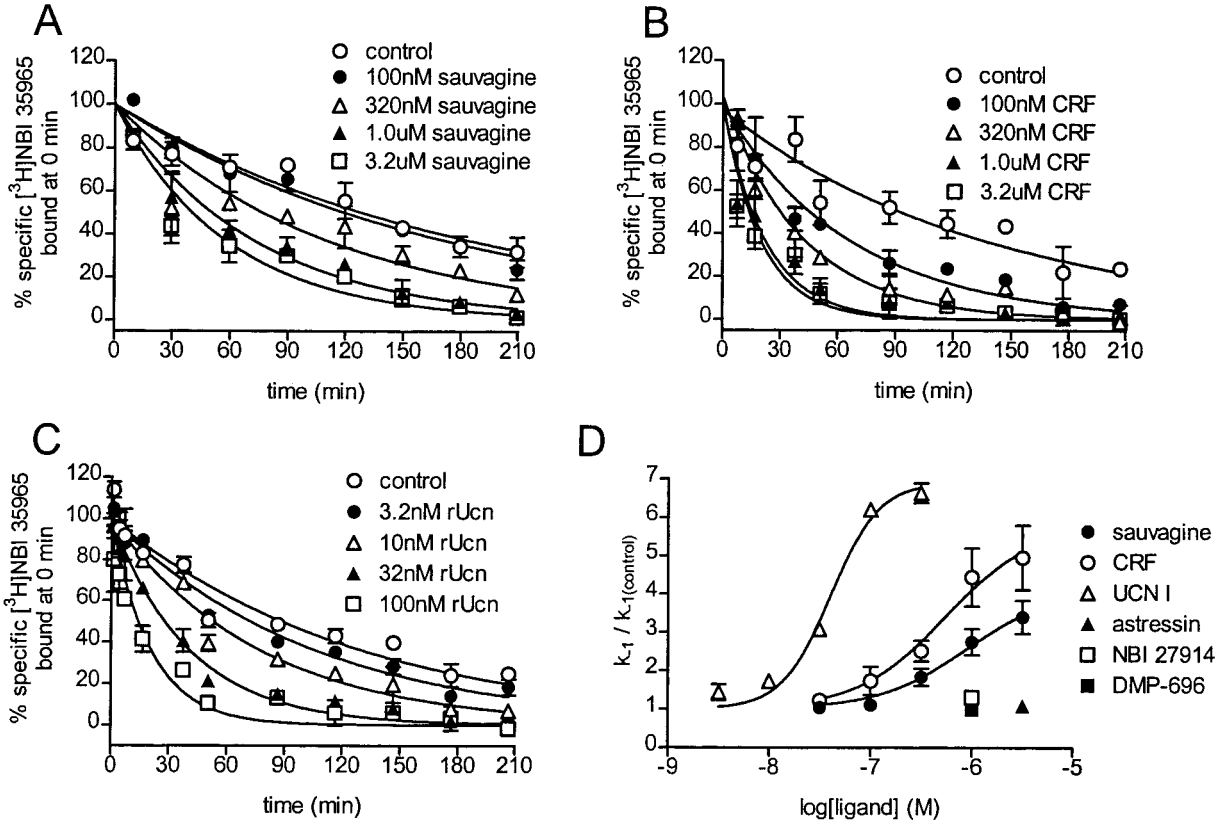


Fig. 5. Modulation of [^{3}H]NBI 35965 dissociation from the R state of the CRF$_1$ receptor by peptide ligands. [^{3}H]NBI 35965 dissociation from L-CRF$_1$ cell membranes was measured in the presence of 30 μM GTPγS, in the presence of sauvagine (A), CRF (B), and UCN I (C). The curves are the best fit 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 μl in assay volume of 200 μl) 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_O (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_O 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 Ehlert (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_d 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 GTPγS, 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

Estimates of peptide ligand affinity, cooperativity, and maximal inhibition of radioligand binding, for allosteric modulation of [^{3}H]NBI 35965 binding to the R state of the CRF_1 receptor

The binding parameters are for the R state of the CRF_1 receptor in L-CRF_1 cell membranes (Fig. 4B, 30 μM GTPgS was included in the assays). The maximal extent of [^{3}H]NBI 35965 inhibition produced by peptide ligand was calculated as the percentage of specific binding inhibited by 10 μM sauvagine, 10 μM CRF, 3.2 μM UCN I, and 3.2 μM astressin. Data for inhibition of [^{3}H]NBI 35965 binding by CRF or UCN I (Fig. 4B) were fitted to the allosteric ternary complex model (eq. 2; Appendix) to estimate the affinity of peptide ligand for the free receptor (not occupied by [^{3}H]NBI 35965, K_I) and to determine the cooperativity (α) between the binding of peptide ligand and [^{3}H]NBI 35965. (The data for sauvagine and astressin could not be reliably fitted using eq. 2, because of the weak maximal inhibition by the ligands.) In this analysis the affinity constant for [^{3}H]NBI 35965 was fixed at the value measured in saturation experiments (1.80×10^9 M^{-1}).

Ligand	% Maximal Inhibition	pK_I ($1/K_I$)	α
		nM	
Sauvagine	13 ± 2	N.D.	N.D.
CRF	29 ± 4	6.57 ± 0.05[a] (270)	0.16 ± 0.04[c]
UCN I	40 ± 7	8.81 ± 0.15[b] (1.6)	0.22 ± 0.02[c]
Astressin	1 ± 4	N.D.	N.D.

N.D., not determined.

[a+b] The affinity of agonists fitted by eq. 2 was not significantly different from the affinity measured by inhibition of ^{125}I-astressin binding ([a]p = 0.58, pK_i = 6.68 ± 0.09 for CRF; [b]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).

[c] The α 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).

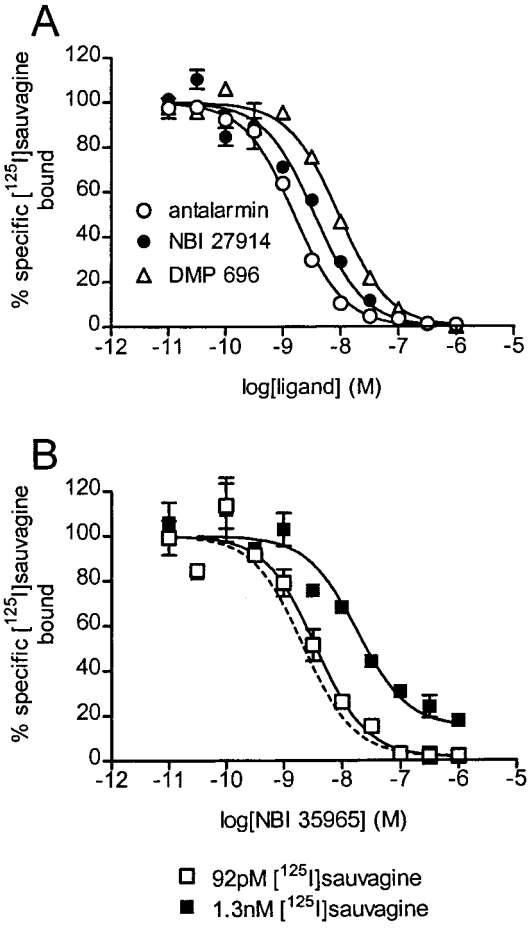


Fig. 6. Inhibition of ^{125}I-sauvagine binding to the RG state of the CRF_1 receptor by nonpeptide antagonists. A, inhibition of binding of low ^{125}I-sauvagine concentrations (79–94 pM) by antalarmin, NBI 27914, and DMP-696, measured as described under *Materials and Methods*. The curves are fits to a single-affinity state inhibition model. [A two-site model did not significantly improve the goodness of fit ($p > 0.05$).] B, inhibition of a low (92 pM) and high (1.3 nM) concentration of ^{125}I-sauvagine binding by NBI 35965. 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 for the high ^{125}I-sauvagine concentration were also fitted to the allosteric ternary complex model (eq. 1), for which the fitted curve is superimposable on the fit to the single-affinity state inhibition model. The mean of the fitted value to eq. 1 were as follows: α = 0.0056 ± 0.0012 and pK_N = 9.15 ± 0.06. These fitted mean parameters for the high concentration of ^{125}I-sauvagine were used to simulate a ^{125}I-sauvagine versus NBI 35965 inhibition curve for the low concentration of ^{125}I-sauvagine (dashed line). 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 or four times with similar results.

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 R_O state as well as the RG state by ^{125}I-sauvagine.

Modulation of Equilibrium Peptide Agonist Binding to the R_O State of the CRF_1 Receptor by Nonpeptide Antagonists. A minor fraction of the CRF_1 receptor population in L-CRF_1 cell membranes (16%) exists in a conformation that binds agonists with high affinity, but which is insensitive to GTPγS (termed R_O).[1] The pharmacological profile of nonpeptide antagonist activity at this state was measured by inhibition of ^{125}I-sauvagine binding to L-CRF_1 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 R_O States of the CRF_1 Receptor. The nonpeptide antagonist affinity for these three states of the CRF_1 receptor was compared using the K_i value for inhibition of [^{3}H]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 R_O for NBI 35965; Table 1). The nonpeptide antagonist affinity for R, RG, and R_O 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 R_O (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

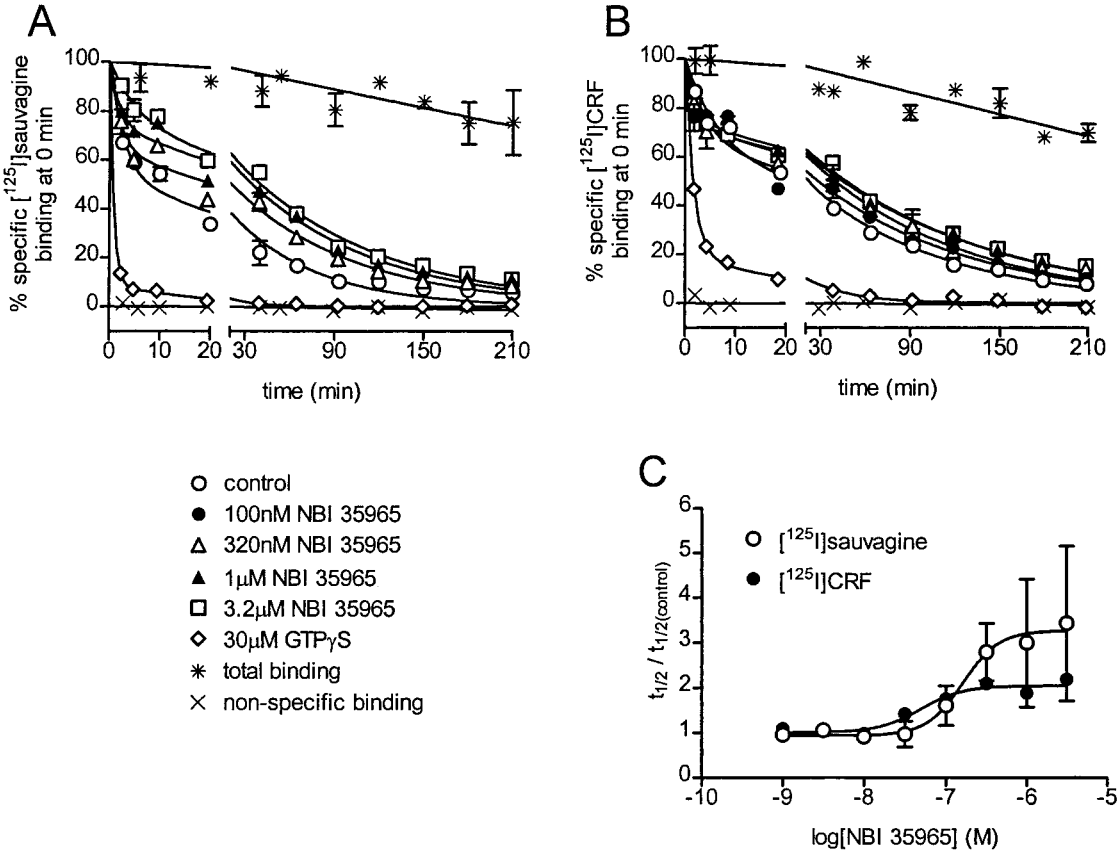


Fig. 7. Modulation of radiolabeled agonist dissociation from the RG state of the CRF_1 receptor by NBI 35965. Dissociation of ^{125}I-sauvagine (A) and ^{125}I-CRF (B) from L-CRF_1 cell membranes was measured as described under *Materials and Methods*, in the absence of antagonist or in the presence 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 monoexponential 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 ± 9%, $k_{-1(fast)}$ = 0.20 ± 0.09 min^{-1}, and $k_{-1(slow)}$ = 0.0098 ± 0.0041 min^{-1} and for ^{125}I-CRF were $P_{(fast)}$ = 38 ± 3%, $k_{-1(fast)}$ = 0.24 ± 0.07 min^{-1}, and $k_{-1(slow)}$ = 0.0089 ± 0.00051 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 CRF_1 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 ^{125}I-astressin dissociation. 2) Reciprocally, peptide agonists only partially inhibited [^{3}H]NBI 35965 binding to the R state and accelerated [^{3}H]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 CRF_1 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 ^{125}I-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 [^{3}H]NBI 35965 binding, suggesting that nonpeptide antagonist can bind the receptor saturated with peptide ligand. 3) Nonpeptide antagonist (NBI 35965) accelerated dissociation of ^{125}I-astressin, consistent with nonpeptide antagonist binding the receptor-^{125}I-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 [^{3}H]NBI 35965 dissociation, suggesting peptide agonist binding to the receptor-[^{3}H]NBI 35965 complex.

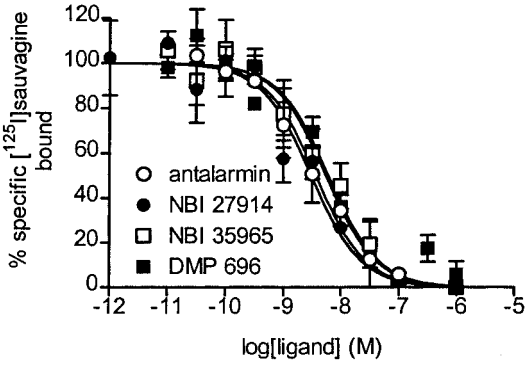


Fig. 8. Inhibition of ^{125}I-sauvagine binding to the R_O state of the CRF_1 receptor by nonpeptide antagonists. Inhibition of ^{125}I-sauvagine binding to L-CRF_1 cell membranes was measured in the presence of 30 μM GTPγS, as described under *Materials and Methods*. Under these conditions the radioligand binds an agonist high-affinity state of the CRF_1 receptor, which is insensitive to GTPγS (R_O). 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.

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 ^{125}I-astressin. This model could explain partial inhibition of ^{125}I-astressin binding by nonpeptide antagonists. However, a number of findings argue against this model. First, the model can only explain partial ^{125}I-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 ^{125}I-astressin. Furthermore, the B_{max} value of [^{3}H]NBI 35965 (6.0 pmol/mg) was similar to that for ^{125}I-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-CRF_1 cell membranes (R, RG, and R_O; 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 [^{3}H]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.[1] However, the model does not allow modulation of [^{3}H]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 CRF_1 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 CRF_1 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 ($\alpha = 0.11$, 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

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 [^{3}H]NBI 35965 binding (Tables 2 and 3; Appendix). This reciprocal relationship has been demonstrated for gallamine and *N*-methylscopolamine at the M_2 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 ^{125}I-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 [^{3}H]NBI 35965 binding to the RG state, to determine whether peptide ligands affect [^{3}H]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 CRF_1 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 K_i value of NBI 27914 for the CRF_1 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 CRF_1 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 CRF_1 receptor, and potentially for other family B GPCRs.

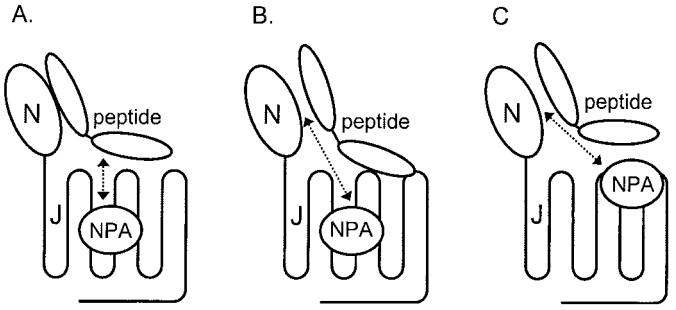


Fig. 9. Potential molecular models of nonpeptide antagonist action at the CRF_1 receptor. These models of nonpeptide ligand and peptide interaction with the receptor are consistent with the data in the present study. Peptide ligand is assumed to consist of two binding regions, which interact with two corresponding binding domains of the receptor (the N- and the J-domain). Nonpeptide antagonist (NPA) is assumed to bind only the J-domain. The dashed line indicates an allosteric interaction between spatially distinct binding sites. A, nonpeptide antagonist allosterically inhibits peptide binding to the J-domain. B, nonpeptide antagonist allosterically inhibits peptide binding to the N-domain. C, nonpeptide antagonist competitively inhibits peptide binding to the J-domain and allosterically inhibits peptide binding to the N-domain. An allosteric component was included in each mechanism because allosteric inhibition was demonstrated for the R state. Note that the figure is a schematic representation and so may not accurately reflect the precise location of the receptor's binding sites.

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 CRF_1 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; Ehlert, 1988; Lazareno and Birdsall, 1995) shown in Scheme 1.

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

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

where $[R_{TOT}]$ is the total receptor concentration. The equation describing the effect of L on the binding of N is as follows:

$$[NR] + [NRL] = [R_{TOT}]\frac{[N]K_N(1 + \alpha[L]K_L)}{1 + [N]K_N(1 + \alpha[L]K_L) + [L]K_L} \quad (2)$$

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:

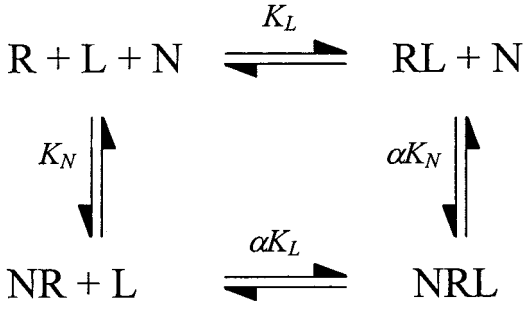
$$[RL^*] + [NRL^*] = [R_{TOT}]\frac{[L^*]K_{L^*}(1 + \beta[N]K_N)}{1 + [L^*]K_{L^*}(1 + \beta[N]K_N) + [L]K_L(1 + \alpha[N]K_N) + [N]K_N} \quad (3)$$

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 obtain estimates of K_N and α (fitted values in Table 1). The fitted pK_N value was in good agreement with the pK_i value of each compound for displacing [^{3}H]NBI 35965 binding (Table 1). The α value was similar for all four antagonists (0.54–0.65; Table 1), indicating a similar extent of negative cooperativity for the ligands. The negative cooperativity was weak: the α value of 0.65 for NBI 35965 indicates that binding of the ligand reduces the affinity of ^{125}I-astressin from 70 to 110 pM.

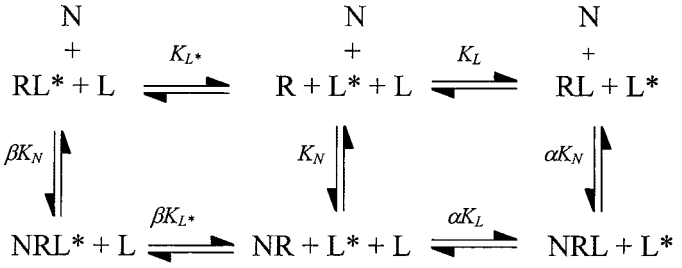
The allosteric effect of NBI 35965 on peptide agonist binding to the R state was quantified by fitting the data of Fig. 2 to eq. 3. The data for agonist binding alone and in the presence of the three concentrations of NBI 35965 were analyzed simultaneously using SigmaPlot 2000 (SPSS Science, Chicago, 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 (α values of 0.11–0.33) than between NBI 35965 and the peptide antagonist astressin (α = 0.65; Table 1). In addition, α 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 α 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 [^{3}H]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 α values for CRF and UCN I versus [^{3}H]NBI 35965 binding (0.16 and 0.22, respectively; Table 3) were in good agreement with the α 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 [^{3}H]NBI 35965 binding (pK_L values of 6.57 and 8.81; Fig. 4B; Table 3) were in good agreement with the pK_i values obtained from inhibition of ^{125}I-astressin binding to the R state (6.68 and 8.81).[1] Unfortunately the inhibition of [^{3}H]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 [^{3}H]NBI 35965 binding (Fig. 4B; Table 3) could be due to the high dose of [^{3}H]NBI 35965 used relative to its K_d value (2.8–6.2-fold the K_d 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_d value decreases the maximal inhibition of radioligand binding by allosteric ligand As a result, the use of high [^{3}H]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 com-



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 α. (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. K_{L^*} is the equilibrium association constant of L* (^{125}I-astressin) binding to R, and β is the cooperativity between L* and N binding to R.

plex (Lazareno and Birdsall, 1995). NBI 35965 accelerated ^{125}I-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 ($p\alpha K_N$) 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 Birdsall, 1995). This value can then be compared with the $p\alpha K_N$ value measured by inhibition of equilibrium ^{125}I-astressin binding (calculated from the fitted values of α 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 Birdsall, 1995). We did not attempt to determine $p\alpha K_N$ from the ^{125}I-astressin dissociation assay, because NBI 35965 associates slowly with the receptor ($t_{1/2}$ of 15 min for association of 2.5 nM [^{3}H]NBI 35965, compared with a $t_{1/2}$ of 24 min for dissociation of ^{125}I-astressin). In addition, this analysis can only be applied if the radioligand dissociates monophasically, whereas ^{125}I-astressin dissociation was biphasic. These considerations notwithstanding, the $p\alpha K_N$ value calculated from equilibrium binding (8.55; αK_N = 2.8 nM) was within the effective concentration range of NBI 35965 for accelerating ^{125}I-astressin dissociation (Fig. 1C). This finding is reasonably consistent with the hypothesis that the same mechanism underlies both the modulation of ^{125}I-astressin dissociation and equilibrium ^{125}I-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 [^{3}H]NBI 35965 from the R state of the CRF_1 receptor (Fig. 5). Peptide agonists accelerated [^{3}H]NBI 35965 dissociation from the R state. The pEC_{50} value of sauvagine and CRF for increasing k_{-1} of [^{3}H]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 $p\alpha K_L$ (see above), because it is likely that the high effective concentrations of peptide rapidly associated with the receptor, and dissociation of [^{3}H]NBI 35965 was slow ($t_{1/2}$ of 80min). The pEC_{50} values for sauvagine and CRF are in reasonable agreement (within 1.5- and 3.2-fold, respectively) with the $p\alpha K_L$ values calculated for modulation of equilibrium [^{3}H]NBI 35965 binding (6.07 and 5.88, respectively). [The equilibrium $p\alpha K_L$ value was calculated using the pK_i from inhibition of ^{125}I-astressin binding[1] and α (Table 2 for sauvagine; Table 3 for CRF)]. The reasonable agreement between $1/\alpha K_L$ from equilibrium and kinetic assays suggest that the same allosteric mechanism underlies regulation of equilibrium [^{3}H]NBI 35965 binding and [^{3}H]NBI 35965 dissociation. However, for CRF and sauvagine we could not determine whether the pEC_{50} value for modulation of [^{3}H]NBI 35965 dissociation better matched the equilibrium K_L value rather than the αK_L value, given the small degree of negative cooperativity between NBI 35965 and the peptides and the accumulated error in the equilibrium estimate of αK_L (from α and K_L). For UCN I, the concentration-response relationship for increasing [^{3}H]NBI 35965's k_{-1} was steep (Fig. 5C; slope factor of 1.93) and the pEC_{50} (7.33) was less than the $p\alpha K_L$ value calculated for modulation of equilibrium [^{3}H]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 [^{3}H]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 [^{3}H]NBI 35965 binding. This reciprocal modulation provides strong evidence for the allosteric ternary complex model (Trankle 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 ^{125}I-sauvagine (99% inhibition) and ^{125}I-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; Ehlert, 1988). When the ^{125}I-sauvagine dose was increased to 1.3–2.1 nM (30–49-fold the K_d), NBI 35965 incompletely inhibited radioligand binding (Fig. 6B), suggestive of an allosteric interaction between NBI 35965 and ^{125}I-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_N = 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 ^{125}I-sauvagine versus NBI 35965 inhibition curve for the low concentration of ^{125}I-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 ^{125}I-sauvagine concentration. Almost all binding is displaced, according to this

model, because the negative cooperativity is high, and the concentration of radioligand (realative to its K_d) is low.

Acknowledgments

We gratefully acknowledge Xin-Jin Liu for synthesis of ^{125}I-astressin and Anil Pahuja for technical assistance.

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