Naunyn-Schmiedeberg's Arch Pharmacol (2001) 362 : 28–33
DOI 10.1007/s002100000327

ORIGINAL ARTICLE

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Characterisation and comparison of novel ligands for the nociceptin/orphanin FQ receptor

Received: 4 May 2000 / Accepted: 24 July 2000 / Published online: 21 September 2000
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Abstract Studies of nociceptin/orphanin FQ (NC) have been hampered by the paucity of available ligands with activity at the nociceptin receptor (NCR). In this study we have compared the agonist profile of NC and a novel NCR agonist, Ro65-6570, in a series of radioligand binding studies and effects on forskolin-stimulated cAMP formation in Chinese hamster ovary (CHO) cells expressing the recombinant human NCR (CHO_{hNCR}). In addition, we report the effects of three antagonists, [Nphe^1]NC(1–13)NH_2, J-113397 and III-BTD, on these responses. In radioligand binding studies Ro65-6570, [Nphe^1]NC(1–13)NH_2, J-113397 and III-BTD displaced [^{3}H]NC with similar pK_i values (8.4–8.8). This compares with a pK_D of 10.2 for NC in a direct saturation experiment. [Nphe^1]NC(1–13)NH_2 and J-113397 showed at least 100-fold selectivity over classical opioid receptors. Both NC and Ro65-6570 produced a concentration-dependent inhibition of cAMP formation with pEC_{50} values of 9.56± 0.06 and 8.68±0.04, respectively. Maximum inhibition achieved was 100%. [Nphe^1]NC(1–13)NH_2, J-113397 and III-BTD produced a parallel rightward shift in the concentration-response curves to both NC and Ro65-6570 with pK_B values of ~6.5, ~7.5 and ~7.7, respectively. Importantly, all three antagonists were devoid of residual agonist activity. Collectively, these data indicate the value of Ro65-6570, [Nphe^1]NC(1–13)NH_2, J-113397 and III-BTD in studies of the physiological role played by NC. However, due to the relatively poor selectivity of Ro65-6570 and III-BTD caution should be exercised when using tissues that co-express μ-opioid receptors.

Key words Nociceptin · Orphanin F/Q · Nociceptin receptor agonist Ro65-6570 · Nociceptin receptor antagonists · [Nphe^1]NC(1–13)NH_2 · J-113397 and III-BTD · Radioligand binding · cAMP formation

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Introduction

Nociceptin/orphanin FQ is the endogenous ligand for the orphan opioid receptor-like receptor (Meunier et al. 1995; Reinscheid et al. 1995) referred to in the remainder of this paper as nociceptin (NC) and nociceptin receptor (NCR). NCR is a member of the G protein receptor superfamily whose activation leads to reduced cAMP formation (Meunier et al. 1995; Reinscheid et al. 1995), an enhanced outward K^+ conductance (Connor et al. 1996a; Vaughan et al. 1997) and closing of voltage-sensitive Ca^{2+} channels (Connor et al. 1996b; Knoflach et al. 1996). In the central nervous system NC has been reported to have effects on pain perception and analgesia, anxiety, locomotion, motivation and feeding. In the periphery, this peptide inhibits neurogenic contractions in various tissues and affects the function of cardiovascular and renal systems (see for recent reviews Calo' et al. 2000b; Harrison and Grandy 2000).

To date, several compounds have been identified with activity at NCR but their actions are variable and appear dependent on the tissue under study. These compounds include naloxone benzoylhydrazone (Nicholson et al. 1998; Noda et al. 1998; Okawa et al. 1998), Ac-RYYRWK-NH_2 (Dooley et al. 1997) and [Phe^{1}ψ(CH_2NH)Gly^2]NC(1–13)NH_2 (Calo' et al. 1998; Guerrini et al. 1998; Okawa et al. 1999). Since peptides represent the major class of NCR ligands, peptidase-dependent degradation is an important experimental problem (Montiel et al. 1997). Whilst the use of peptidase inhibitors can prevent this degradation, there would be major advantages in the development of

non-peptide selective ligands. This is important not only experimentally but also for future development of clinically useful compounds. Recently, (*RS*)-8-acenaphthen-1-yl-phenyl-1,3,8-triaza-spirol[4,5]decan-4-one (compound 1a or Ro65-6570) was identified as a non-peptide NCR agonist with high affinity and moderate selectivity over the opioid (μ, δ, κ) receptors (Wichmann et al. 1999). In this study, we have evaluated the in vitro pharmacological profile of Ro65-6570 and compared this with NC in Chinese hamster ovary (CHO) cells expressing the human NCR (CHO_{hNCR}). In addition, we have further probed these responses with a range of novel peptide and non-peptide NCR antagonists: [$Nphe^1$]NC(1–13)NH_2 ($Nphe^1$; Calo' et al. 2000a; Hashimoto et al. 2000), [(3*R*,4*R*)-1-cycloptylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (J113397; Kawamoto et al. 1999; Ozaki et al. 2000), and (3*S*,6*S*,9*R*)-2-oxo-3-amino-7-thia-1-aza-bicyclo [4,3,0]nonane-9-carboxylic acid(III-BTD; Becker et al. 1999).

Materials and methods

Materials

Chemicals and their sources were as follows; NC, $Nphe^1$ and J-113397 were synthesized at one of our institutes as previously described (Guerrini et al. 1997, 2000; Bigoni et al. 2000a). Ro65-6570 was synthetized as reported by Roever et al. (2000). Briefly, reaction of acenaphthen-1-one (which was synthesised as described in Bryce et al. 1996; 6.7 mmol), 1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one (commercially available from Fluka; 6.7 mmol) and titanium (IV) isopropoxide (8.4 mmol) in tetrahydrofuran (15 ml) and subsequent reduction (after evaporation of the tetrahydrofuran) with sodium cyanoborohydride (6.7 mmol) in ethanol (15 ml) yielded, after column chromatography, a pale brown foam which was then crystallized as yellowish hydrochloride salt from methanol/HCl and diethyl ether. The purity of the compound was assessed by HPLC analysis performed on a C18 chromatography column and the structure of the compound verified by MALDI-TOF mass spectrometry and NMR spectroscopy.

III-BTD was purchased from Neosystem (Strasbourg, France). All cell culture media and supplements were from Gibco (Paisley, UK). [2,8-^{3}H]cAMP (28.4 Ci/mmol), [leucyl-^{3}H]NC (151 Ci/mmol) and [^{3}H]diprenorphine ([^{3}H]DPN; 56 Ci/mmol) were purchased from NEN Dupont (Boston, Mass., USA) and Amersham Pharmacia Biotech (Buckinghamshire, UK), respectively. All other reagents were of the highest purity available. CHO_{hNCR} cells were from Dr. F. Marshall and Mrs. N. Bevan of Glaxo-Wellcome (Stevenage, Herts, UK). CHO cells expressing the rat μ- or κ-opioid receptor ($CHO_{rμ}$, $CHO_{rκ}$) were from Dr. D.K. Grandy (Vollum Institute, Ore., USA). CHO cells expressing the mouse δ-opioid receptor ($CHO_{mδ}$) were from Dr. L.A. Devi (New York University, N.Y., USA).

Cell culture and membrane preparation

CHO_{hNCR} cells were maintained in DMEM: Ham F12 (50:50) containing 5% FCS, 2 mM glutamine, 50 IU/ml penicillin (P), 50 μg/ml streptomycin (S), 200 μg/ml hygromycin B and 200 μg/ml G418. $CHO_{rμ}$, $CHO_{rκ}$ and $CHO_{mδ}$ cells were grown in Ham F12 containing 10% FCS, 50 IU/ml P, 50 μg/ml S, 200 μg/ml G418 and 2.5 μg/ml amphotericin B. Cell cultures were kept at 37°C in 5% CO_2/humidified air. When confluency was reached (3–4 days), cells were harvested for use by the addition of 0.9% saline containing HEPES (10 mM)/EDTA (0.05%). Membranes were prepared from cell suspensions in either Tris (50 mM), $MgSO_4$ (5 mM), pH 7.4, for CHO_{hNCR} or 50 mM Tris, pH7.4, for CHO-opioid expression systems. Cell suspensions were homogensied on ice, then centrifuged at 13,500 rpm for 10 min at 4°C. This procedure was repeated twice more. The resulting membrane pellet was resuspended as appropriate for each experiment (see below). All binding studies were performed using fresh membranes.

Binding assays

[Leucyl-^{3}H]NC. Approximately 5 μg membrane protein was incubated in 0.5 ml of homogenisation buffer containing 10 μM of peptidase inhibitors (captopril, amastatin, bestatin and phosphoramidon) and 0.5% BSA for 30 min at room temperature. In saturation experiments, various concentrations of [leucyl-^{3}H]NC (0.001–1 nM) were used. In displacement experiments 0.2 nM [leucyl-^{3}H]NC was used. In all studies non-specific binding (NSB) was defined in the presence of 1 μM unlabelled NC. Following incubation, bound and free radioactives were separated by vacuum filtration using a Brandel cell harvester. Harvester papers (Whatman GF/B) were soaked in polyethylenimine (0.5%) to reduce NSB, and loaded onto the harvester wet.

[^{3}H]Diprenorphine. This is essentially as described above. Approximately 100 μg membrane protein was incubated in 1.0 ml of homogenization buffer containing the peptidase inhibitor cocktail and BSA for 60 min at room temperature. Displacement studies were performed using 0.3–0.5 nM [^{3}H]diprenorphine ([^{3}H]DPN) and varying concentrations of unlabelled displacer. NSB was defined in the presence of 10 μM naloxone. Bound and free radioactivity were separated as described above.

Cyclic AMP assay

CHO cell suspensions were washed twice with and resuspended in Krebs/HEPES buffer of the following composition (mM): Na^+ (143.3), K^+ (4.7), Ca^{2+} (2.5), Mg^{2+} (1.2), Cl^- (125.6), $H_2PO_4^{2-}$ (1.2), SO_4^{2-} (1.2), glucose (11.7), and HEPES (10), BSA 0.5%, pH 7.4 with 10 M NaOH. cAMP formation was measured in 0.3 ml volumes of whole cell suspensions in the presence of isobutylmethylxanthine (IBMX; 1 mM) and forskolin (FSK; 1 μM). Peptidase inhibitors were not used as they did not influence the response to NC (data not shown). NC or Ro65-6570 was included in various concentrations in order to obtain a full concentration-response curve. As antagonists, 10 μM $Nphe^1$, 0.1 μM J113397 and 1 μM III-BTD were used. After 15 min of incubation in the presence or absence of the antagonists at 37°C, reactions were terminated and cAMP extracted by the addition of HCl (10 M). For experiments with J113397 cells were preincubated for 15 min. cAMP was assayed as described (Okawa et al. 1999).

Data analysis

Data are expressed as either means ± SEM or means (95% CL). In binding studies, concentration of displacers producing 50% displacement of specific binding (pIC_{50}) was corrected for the competing mass of [^{3}H]DPN or [leucyl-^{3}H]NC to yield pK_i. In these calculations K_d values for [^{3}H]DPN binding to $CHO_{rμ}$ (0.24 nM; Smart et al. 1997), $CHO_{rκ}$ (0.16 nM; Hirst et al. 1997) and $CHO_{mδ}$ (0.67 nM; Hirst et al. 1998) were as previously reported by us and that for [leucyl-^{3}H]NC was from a log-transformation of the specific binding data. The log-molar concentration of each agonist producing half-maximal inhibition of cAMP formation (pEC_{50}) was obtained by computer-assisted curve fitting of individual curves. All curve fitting was performed using PRIZM-V2.0 (GraphPad software; San Diego, Calif., USA). Antagonist affinities are expressed in terms of pK_B, which is the negative logarithm to base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original sub-

maximal response. pK_B was calculated using the following formula: pK_B=-log{(CR-1)/[antagonist]}, where CR is the ratio of the EC_{50} of the agonist in the presence and absence of antagonist, assuming a slope value =1.

Results

Binding assays

[Leucyl-^{3}H]NC binding to CHO_{hNCR} was concentration-dependent and saturable (Fig. 1A), with B_{max}, pK_D and slope factors of 1913±24 fmol/mg protein, 10.15±0.05 and 1.35±0.08, respectively (n=3). pK_i values of Ro65-6570 and the three NC antagonists to CHO_{NCR} (Fig. 1B), and to $CHO_{r\mu/r\kappa/m\delta}$, are shown in Table 1 with the opioid binding data for Nphe^1 taken from Calo' et al. (2000a) for comparison. The degree of NCR selectivity was variable with III-BTD displaying the poorest selectivity. Ro65-6570 displayed poor selectivity over the μ receptor. In contrast, J-113397 and Nphe^1 displayed at least 100-fold selectivity over classical opioid receptors.

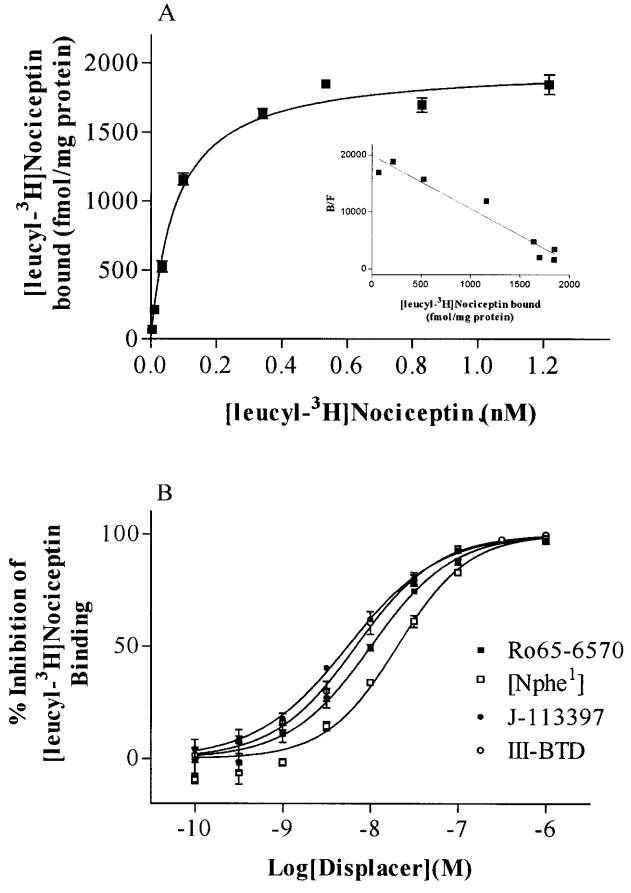

Fig. 1 **A** [Leucyl-^{3}H]nociceptin binding to CHO_{hNCR} is concentration-dependent, saturable and **B** displaced by Ro65-6570, Nphe^1, J-113397 and III-BTD. Binding slope factors for Ro65-6570, Nphe^1, J-113397 and III-BTD were 0.94±0.03, 1.07±0.07, 0.83±0.04 and 0.97±0.13, respectively. In **A** the *insert* shows a Scatchard transform of data where *B/F*=Bound/Free. Data are means ± SEM (n=3–4)

Cyclic AMP assay

Ro65-6570 and NC produced a concentration-dependent inhibition of forskolin-stimulated cyclic AMP accumulation in CHO_{hNCR} cells (Fig. 2). The pEC_{50} and maximal inhibition (E_{max}) values of Ro65-6570 were 8.68±0.04 and 102±0.6 (n=11), while those values of NC were 9.56±0.06 and 104.1±0.4 (n=7). Nphe^1, J-113397 and III-BTD are devoid of significant residual agonist activity. All compounds shifted to the right the concentration-response curve for both NC and Ro65-6570 without significantly affecting the maximal response, consistent with a competitive mode of action. Data for Ro65-6570 are shown in Fig. 3. The estimated pK_B values of the three antagonists (against both Ro65-6570 and NC) are shown in Table 2. The effects of Nphe^1 and J-113397 on NC response are from Hashimoto et al. (2000) and Bigoni et al. (2000a) and are included for comparison. It should be noted that for the effects of Nphe^1 a full Schild analysis has previously been performed (Calo' et al. 2000a) and as such the quoted pK_B approximates the pA_2.

Discussion

We have demonstrated that a novel NCR ligand, Ro65-6570, acts as a full agonist in CHO_{hNCR} cells. Ro65-6570 was about sevenfold less potent than the natural ligand, NC. However, the selectivity of this compound is not great, especially when compared to the μ receptor (five fold). This is in line with the initial report of Wichmann et al. (1999) where tenfold selectivity (in a recombinant system) was reported. Ro65-6570 is in fact a racemic mixture with the (R) isomer having slightly increased (16-fold) selectivity. Clearly the use of this compound will be limited in tissues that co-express μ-opioid receptors. Subsequent to the description of Ro65-6570, other non-peptide NCR agonists have been described by Roche (Jenck et al. 2000; Roever et al. 2000). Among

Table 1 pK_i values for Ro65-6570, [Nphe^1]NC(1–13)NH_2, J-113397 and III-BTD in CHO cell membranes expressing recombinant NCR or opioid receptors. Data are means ± SEM (n=3–6; *Nphe^1* [Nphe^1]NC(1–13)NH_2)

Receptor Radioligand	NCR [Leucyl-^{3}H]NC	μ [^{3}H]DPN	δ [^{3}H]DPN	κ [^{3}H]DPN
Ro65-6570	8.60±0.06	7.89±0.07	6.52±0.04	6.83±0.07
Nphe^1	8.37±0.02	<5	<5	5.96
J-113397	8.82±0.02	6.67±0.05	<5	6.64±0.04
III-BTD	8.74±0.08	8.05±0.05	7.22±0.13	7.94±0.11

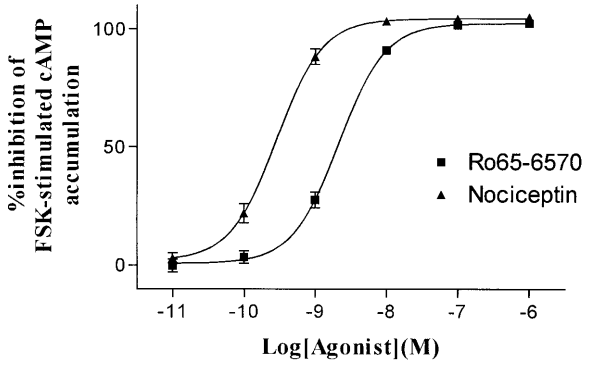

Fig. 2 Ro65-6570 and NC produce a concentration-dependent inhibition of forskolin-stimulated cAMP formation in CHO_{hNCR} cells. Data are means ± SEM (*n*=7–11)

these, Ro64-6198 (Jenck et al. 2000) appears to be the most interesting compound. Ro64-6198 is a high affinity (pK_i 9.41) and highly selective (120-, 229- and 3548-fold over μ, κ and δ, respectively) ligand for the NCR. Ro64-6198 is a full agonist for stimulation of [^{35}S]GTPγS binding and inhibition of forskolin-stimulated cAMP formation. In addition, the authors completed a number of in vivo behavioural tests and showed an impressive anxiolytic profile for this compound comparable to benzodiazepines (Jenck et al. 2000). In parallel with the search for selective NCR agonists the identification of antagonists is of great importance for NC research as these tools are vital in defining the participation of the NC/NCR system in specified biological actions. Several candidate molecules have been described including naloxone benzoylhydrazone and [$Phe^1\psi(CH_2NH)Gly^2$]NC(1–13)NH_2; however, these compounds have limited use due to activity at classical opioid receptors (Berzetei-Gurske et al. 1995) and variable efficacy (Calo' et al. 2000b), respectively.

Recently we described a novel, pure but weak (pA_2 ~6), selective NCR antagonist, [$Nphe^1$]NC(1–13)NH_2 (Calo' et al. 2000a, 2000b; Guerrini et al. 2000). The antagonistic activity of [$Nphe^1$]NC(1–13)NH_2 has been extensively confirmed (Rizzi et al. 1999; Berger et al. 2000; Calo' et al. 2000a; Sbrenna et al. 2000). [$Nphe^1$]NC(1–13)NH_2 is also active in vivo where it prevents the pronociceptive and antimorphine actions of NC in the mouse tail withdrawal assay (Calo' et al. 2000a) and the stimulatory effect of NC on rat food intake (Polidori et al. 2000). Two other antagonists have recently been described: a further poorly selective peptidic compound, III-BTD (Becker et al. 1999; Bigoni et al. 2000b), and the selective non-peptide molecule J-113397 (Kawamoto et al. 1999; Ozaki et al. 2000). In this study J-113397 clearly acts as a potent NCR antagonist with a pK_B value (7.5) very similar to the pIC_{50} reported by Ozaki et al. (2000) in the same assay (7.6), and to those we found in NC-sensitive isolated tissues from various species (7.7–8.0; Bigoni et al. 2000a). To date no data are available describing the in vivo activities of peptide III-BTD or J-113397.

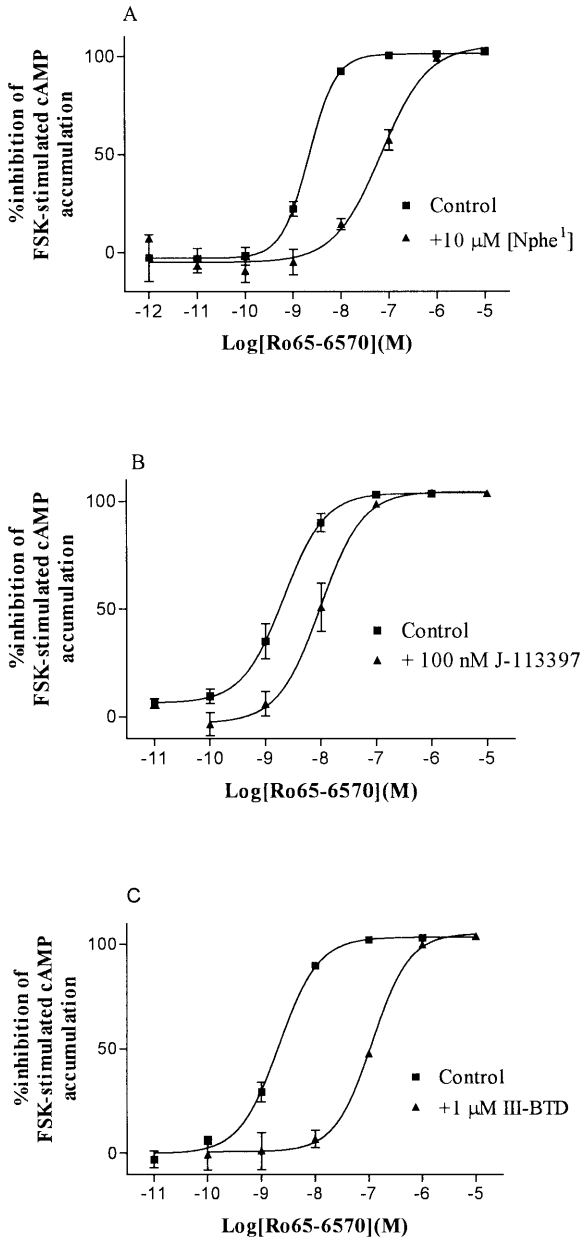

Fig. 3 Effects of **A** 10 μM [$Nphe^1$]NC(1–13)NH_2 ($Nphe^1$), **B** 100 nM J113397, and **C** 1 μM III-BTD on Ro65-6570 inhibition of forskolin-stimulated cAMP formation. All antagonists were devoid of residual agonist activity and produced a rightward shift in the Ro65-6570 concentration-response curve. Data are means ± SEM (*n*=3–6)

Table 2 Antagonist potencies (pK_B) of [$Nphe^1$]NC(1–13)NH_2, J-113397 and III-BTD on Ro65-6570- and NC-mediated inhibition of cyclic AMP accumulation. Data are means (95% CL; *n*=3–6)

	[$Nphe^1$]NC(1–13)NH_2	J-113397	III-BTD
Nociceptin	6.23 (0.22)	7.52 (0.55)	7.49 (0.13)
Ro65-6570	6.50 (0.37)	7.54 (0.29)	7.73 (0.3)

We have examined the effects of the three novel antagonists described above against NC and Ro65-6570 in CHO cells expressing the hNCR. All compounds behave

as competitive antagonists. J-113397 along with III-BTD clearly display high potency (pK_B ~7.5–7.7), which is a marked improvement over Nphe[1] which is about 30-fold less potent. Thus the rank order of potency of these antagonists at the human NCR is: J113397 = III-BTD > Nphe[1]. All these antagonists displayed similar pK_B values when tested against NC or Ro65-6570, suggesting that both the peptide and the non-peptide agonist are likely to activate the hNCR via interaction with the same receptor site.

Careful comparison of the binding and functional data for the ligands used in this study reveals a discrepancy. It appears that for agonists (NC and Ro65-6570) binding affinity and potency values are similar. For antagonists (Nphe1, J113397 and IIIBTD) potency/affinity ratios are >10. In interpreting these differences the following points should be considered: (1) the preparation used in binding assays (cell membranes) is different from that used for the functional assay (intact cells); (2) the buffers used and the temperature for the two assays are different; (3) our CHO_{hNCR} cells express high levels of receptors (B_{max} ~2000 fmol/mg protein).

In general, receptor binding affinities for antagonists should predict their potencies in functional assays. However, in our assays due to differences in the preparations, buffer system and temperature, the occupancy curves (in binding studies) are shifted to the left compared to the response curves (in functional studies), yielding potency/affinity ratios in excess of 10. In contrast, the high level of receptor expression suggests the presence of a large receptor reserve and hence agonists induce the maximal effect by occupying just a small fraction of the available receptors, i.e., for agonists the occupancy curves are shifted to the right compared to the response curves. Since for agonists the final result depends on the combination of the preparation, buffer and temperature differences (that shift the functional curves to the right) with the receptor reserve (that shifts the functional curves to the left), the net result is that for agonists the occupancy curves are (apparently) superimposable with the response curve and hence the potency/affinity ratios for agonists are close to 1. This interpretation is corroborated by the fact that [F/G]NC(1–13)NH_2, which is a partial agonist at the NCR (Calo' et al. 2000b) but behaves as full agonist in this preparation (Okawa et al. 1999), displays a potency/affinity ratio similar to those of antagonists.

Collectively, our data demonstrate that Ro65-6570 is a potent NCR agonist which has high affinity and moderate selectivity for hNCR. Therefore, Ro65-6570 in combination with the growing number of novel NCR ligands [particularly the highly selective and potent non-peptide ligands Ro64-6198 (agonist) and J-113397(antagonist)] represent important new tools for studies of the physiopathophysiological role(s) played by the NC/NCR system.

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