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European Journal of Pharmacology 402 (2000) 45–53

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In vitro and in vivo pharmacological characterization of J-113397, a potent and selective non-peptidyl ORL1 receptor antagonist

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Received 10 April 2000; received in revised form 13 July 2000; accepted 17 July 2000

Abstract

1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (J-113397) was found to be the first potent nonpeptidyl ORL1 receptor antagonist (K_i: cloned human ORL1 = 1.8 nM) with high selectivity over other opioid receptors (K_i: 1000 nM for human μ-opioid receptor, > 10,000 nM for human δ-opioid receptor, and 640 nM for human κ-opioid receptor). In vitro, J-113397 inhibited nociceptin/orphanin FQ-stimulated [^{35}S]guanosine 5′-*O*-(γ-thio)triphosphate (GTPγS) binding to Chinese Hamster Ovary (CHO) cells expressing ORL1 (CHO-ORL1) with an IC_{50} value of 5.3 nM but had no effect on [^{35}S]GTPγS binding by itself. Schild plot analysis of the [^{35}S]GTPγS binding assay and cAMP assay using CHO-ORL1 indicated competitive antagonism of J-113397 on the ORL1 receptor. In CHO cells expressing μ-, δ- or κ-opioid receptors, J-113397 had no effects on [^{35}S]GTPγS binding up to a concentration of 100 nM, indicating selective antagonism of the compoud on the ORL1 receptor. In vivo, J-113397, when administered subcutaneously (s.c.), dose-dependently inhibited hyperalgesia elicited by intracerebroventricular (i.c.v.) administration of nociceptin/orphanin FQ in a tail-flick test with mice. An in vitro binding study using mouse brains indicated that J-113397 possesses high affinity for the mouse ORL1 receptor (K_i: 1.1 nM) as well as the human receptor. In summary, J-113397 is the first potent, selective ORL1 receptor antagonist that may be useful in elucidating the physiological roles of nociceptin/orphanin FQ.
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Keywords: Nociceptin/orphanin FQ; ORL1 receptor; Opioid

1. Introduction

Nociceptin, or orphanin FQ (Meunier et al., 1995; Reinscheid et al., 1995), is a 17-amino acid peptide recently identified as a potent endogenous ligand for the ORL1 receptor that shows a high degree of structural homology with the classical μ-, δ- and κ-opioid receptors (Mollereau et al., 1994). Despite the structural similarities between nociceptin/orphanin FQ and other opioid peptides, especially dynorphin A, and between ORL1 and other opioid receptors, nociceptin/orphanin FQ does not interact with classical opioid receptors and, vice-versa, opioid peptides do not interact with the ORL1 receptor (Mollereau et al., 1994; Meunier et al., 1995; Reinscheid et al., 1995). At the cellular level, nociceptin/orphanin FQ has effects similar to those of the classical opioids. The ORL1 receptor is coupled to activation of inwardly rectifying K^+ (K_{IR}) channels (Ikeda et al., 1997) and/or negatively coupled to adenylate cyclase via a pertussis toxin-sensitive G-protein (Chan et al., 1998). A large number of in vivo studies showed a wide variety of biological effects of nociceptin/orphanin FQ. Intracerebroventricular (i.c.v.) administration of nociceptin/orphanin FQ caused suppression of spatial learning (Sandin et al., 1997), stimulation and reduction of locomotor activity (Florin et al., 1996), stimulation of food intake (Pomonis et al., 1996) and

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PII: S0014-2999(00)00520-3

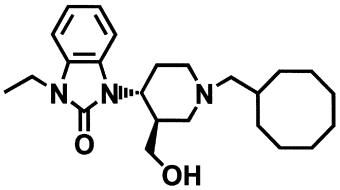

Fig. 1. Structure of J-113397.

anxiolytic effects (Jenck et al., 1997). The role of nociceptin/orphanin FQ in the regulation of pain is controversial. Supraspinal administration of nociceptin/orphanin FQ was reported to produce pronociceptive effects (Meunier et al., 1995; Reinscheid et al., 1995; Calò et al., 1998), inhibit opioid-mediated stress-induced antinociception (Mogil et al., 1996) and enhance formalin-induced pain response (Wang et al., 1999). Rossi et al. (1996) reported that supraspinal injection of nociceptin/orphanin FQ causes both hyperalgesia and analgesia depending on the experimental condition. In contrast, spinal administration of nociceptin/orphanin FQ was reported to produce analgesia (Xu et al., 1996) and inhibit formalin-induced pain response (Erb et al., 1997). Interestingly, Hara et al. (1997) reported that intrathecal injection of nociceptin/orphanin FQ, at an extremely low concentration (10^{-14}–10^{-8} g/kg mouse) compared with that administered in other studies, produces hyperalgesia and allodynia in conscious mice. There were no significant changes in nociceptive threshold in ORL1-deficient mice, making it more difficult to clarify the physiological role of nociceptin/orphanin FQ in pain regulation (Nishi et al., 1997).

The lack of a potent and selective antagonist for ORL1 has hampered determination of the definitive physiological roles of nociceptin/orphanin FQ-ORL1 systems. Recently, we reported that 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (J-113397) is a potent and selective nonpeptidyl ORL1 receptor antagonist (Fig. 1) (Kawamoto et al., 1999; Ozaki et al., 2000). In this report, we describe the results of detailed characterization of J-113397 in in vitro and the in vivo effects of J-113397 on the tail flick test in mice.

2. Materials and methods

2.1. Materials

Nociceptin/orphanin FQ was synthesized in our laboratory. [^{125}I][Tyr^{14}]nociceptin, [^{3}H]diprenorphine, [^{3}H](+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U-69593) and [^{35}S]guanosine 5′-*O*-(γ-thio)triphosphate (GTPγS) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK); [^{3}H][D-Ala^{2}, D-Leu^{5}]enkephalin (DADLE), from NEN Life Science Products (Boston, MA, USA); naloxone, [D-Ala^{2}, *N*-Me-Phe^{4}, Gly-ol^{5}] enkephalin (DAMGO), naltrindole, nor-binaltorphimine (norBNI) and [Phe^{1}Ψ(CH$_2$-NH)Gly^{2}]nociceptin-(1-13)-NH$_2$ from Tocris Cookson (Bristol, UK); DADLE, U-69593 and naloxone benzoylhydrazone from Reseach Biochemicals International (Natrick, MA, USA); and membranes derived from Chinese Hamster Ovary (CHO) cells expressing human μ-, δ- and κ-opoid receptors (CHO-μ, CHO-δ, CHO-κ), from Receptor Biology (Beltsville, MD, USA).

2.2. Cloning of the ORL1 receptor

A 1337 bp ORL1 clone was PCR amplified from human amygdala cDNA (Clonetech, Palo Alto, CA, USA) using a forward primer (GTTGCAGAAGTACCGTACAGA) and a reverse primer (GATGTCACTAGGTCCTCCTC) on the 5′ and 3′ non-coding sequence of human ORL1. The PCR product was cloned into the expression vector pCR3 (Invitrogen, San Diego, CA, USA), and then transfected into CHO-K1 cells. The cells were selected for stable integration of the ORL1 receptor expression vector by adding 1 mg/ml G-418 to the medium and were isolated by single cell cloning. The expression of the receptor was confirmed by both [^{125}I][Tyr^{14}]nociceptin binding study and cyclic AMP measurement.

CHO-K1 cells expressing ORL1 receptor (CHO-ORL1) were grown in Ham's F-12 medium containing 10% fetal bovine serum and 1 mg/ml G-418 at 37°C under atmospheric conditions of 95% air–5% CO_2.

2.3. Competition binding study

Membranes from CHO-ORL1 cells were prepared as previously described (Ozaki et al., 1996). [^{125}I][Tyr^{14}]nociceptin binding assays were conducted in 50 mM HEPES, 10 mM NaCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.1% bovine serum albumin, and 0.025% bacitracin, pH 7.4 using CHO-ORL1 membranes with 50 pM [^{125}I][Tyr^{14}]nociceptin at 37°C for 60 min. [^{3}H]diprenorphine binding assays were conducted in 50 mM Tris, pH 7.4 using CHO-μ with 2 nM radioligand at 25°C for 2 h. [^{3}H]DADLE binding assays were conducted in 50 mM Tris and 5mM $MgCl_2$, pH 7.4 using CHO-δ with 5 nM radioligand at 25°C for 2 h. [^{3}H]U-69593 binding assays were conducted in 50 mM Tris, 10mM $MgCl_2$ and 1 mM EDTA, pH 7.4 using CHO-κ with 2 nM radioligand at 25°C for 2 h. Non-specific binding was determined in the presence of an excess amount of cold nociceptin, naloxone, DADLE, and U-69593, respectively. K_i values were calculated by the equation; $K_i = IC_{50}/(1 + [L]/K_d)$ (Cheng and Prusoff, 1973).

2.4. Measurement of agonist /antagonist activity by [^{35}S]GTPγS binding studies

[^{35}S]GTPγS binding studies were carried out by a minor modification of the method described by Lazareno and

Birdsall (1993). In brief, membranes from CHO-ORL1, or μ-, δ- or κ-opioid receptors were incubated with 400 pM [^{35}S]GTPγS in 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 5 μM GDP, pH 7.4 containing 1.5 mg of wheat germ agglutinin-coated SPA beads (Amersham Pharmacia Biotech) for 2.5 h at 25°C in the presence or absence of various concentrations of an agonist or an antagonist. Membrane-bound radioactivity was detected by scintillation proximetry (Nelson, 1987) with a TopCount microplate scintillation counter (Packard, Meriden, CT, USA).

2.5. Measurement of cyclic amp in CHO-ORL1 cells

CHO-ORL1 cells were preincubated with Locke's buffer, pH 7.4 (154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 10 mM HEPES and 0.3 mM 3-isobutyl-1-methylxanthine) at 25°C for 10 min. The buffer was then changed to the same buffer containing 10 μM forskolin and 0.001–100 nM nociceptin/orphanin FQ with or without various concentrations of J-113397 for 10 min at 25°C. The reaction was stopped by the removal of the reaction mixture followed by the addition of 1% SDS. Intracellular cyclic AMP levels were determined using a BIOTRAK cyclic AMP direct assay kit (Amersham Pharmacia Biotech).

2.6. [^{125}I][Tyr^{14}]nociceptin binding study in mouse brains

Membranes from mouse brains were prepared as previously described (Ozaki et al., 1996). [^{125}I][Tyr^{14}]nociceptin binding assays were conducted in 50 mM HEPES, 10 mM NaCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.1% bovine serum albumin, 0.025% bacitracin, and 100 μM bestatin, pH 7.4 using mouse brain membranes with 50 pM [^{125}I][Tyr^{14}]nociceptin at 25°C for 60 min. Non-specific binding was determined in the presence of an excess amount of cold nociceptin/orphanin FQ. The free and membrane-bound radioligands were separated by filtration using Whatman GF/C glass fiber filters. K_i values were calculated by the equation; $K_i = IC_{50}/(1 + [L]/K_d)$ (Cheng and Prussoff, 1973).

2.7. Autoradiography with [^{125}I][Tyr^{14}]nociceptin in mouse brains

Mice were killed by decapitation and the brains were removed and frozen in dry-ice-isopentane. Coronal brain sections (12 μm) were cut on a cryostat maintained at −20°C. Sections were incubated with 50 pM [^{125}I][Tyr^{14}]nociceptin in Hank's balanced salt solution (HBSS) containing 0.1% glucose and 0.1% bovine serum albumin with or without J-113397 (0.1–100 nM) at 25°C for 60 min, and were then rinsed twice in ice-cold HBSS and rinsed once briefly in deionized water. Sections were then dried and exposed to hyperfilm-βmax (Amersham Pharmacia Biotech).

2.8. Tail-flick test in mice

The response to a thermal nociceptive stimulus was determined using the tail-flick test. Thermal heat was applied to the tail by infrared light using Type 7360, Ugo Basile (Varese, Italy). The intensity of the heat source was adjusted to provide a baseline tail-flick response latency of approximately 8 s, and the cut-off time was set at 15 s.

Male ICR mice (15–25 g) were i.c.v. injected with vehicle or 0.01–1 nmol nociceptin/orphanin FQ for evaluation of the pronociceptive response caused by nociceptin/orphanin FQ. The injections were made directly into the left lateral ventricle through the coronal suture according to the method of Laursen and Belknap (1986). To evaluate the in vivo effects of J-113397, various concentrations (3–30 mg/kg) of J-113397 were injected subcutaneously (s.c.) 10 min prior to administering 0.1 nmol nociceptin/orphanin FQ to the mice. The tail-flick latencies were measured 15 min after administration of the agonist.

2.9. Statistical analysis

Data were expressed as mean ± S.E.M. Statistical comparison was performed using the analysis of variance and post-hoc multiple comparison analysis with a modified *t*-test (Dunnett's). *P*-values of < 0.05 were accepted as significant.

3. Results

3.1. Binding affinity of J-113397 to ORL1 and other opioid receptors

In competition binding assays, J-113397 potently inhibited [^{125}I][Tyr^{14}]nociceptin binding to CHO-ORL1 mem-

Table 1
Binding affinities of J-113397 for the ORL1 receptor, and for μ-, δ- and κ-opioid receptors

	K_i (nM)[a]			
	ORL1[b]	μ[c]	δ[d]	κ[e]
J-113397	1.8 ± 0.24	1000 ± 160	> 10,000	640 ± 87
NalBzoH[f]	38 ± 4.4	1.5 ± 0.13	11 ± 0.13	0.25 ± 0.018
Naloxone	> 10,000	21 ± 2.1	320 ± 47	5.1 ± 0.53
Naltrindole	800 ± 84	210 ± 15	0.32 ± 0.039	17 ± 0.98
norBNI	7900 ± 1700	100 ± 18	30 ± 3.5	2.5 ± 0.093
[F/G]NC(1–13)[g]	1.2 ± 0.12	> 1000	> 1000	> 1000
NC/OFQ[h]	0.44 ± 0.042	> 1000	> 1000	> 1000

[a] Data are mean ± S.E.M. of three experiments.
[b] [^{125}I][Tyr^{14}]nociceptin binding to CHO-ORL1.
[c] [^{3}H]diprenorphine binding to CHO-μ.
[d] [^{3}H]DADLE binding to CHO-δ.
[e] [^{3}H]U-69593 binding to CHO-κ.
[f] Naloxone benzoylhydrazone.
[g] [Phe^{1}Ψ(CH_2-NH)Gly^{2}]nociceptin-(1-13)-NH_2.
[h] Nociceptin/orphanin FQ.

branes with a K_i value of 1.8 ± 0.24 nM (Table 1) but did not inhibit [^{3}H]diprenorphine, [^{3}H]DADLE and [^{3}H]U-69593 binding to CHO-μ, -δ and -κ membranes, respectively, at concentrations up to 100 nM. The K_i values for J-113397 calculated from these results were 1000 ± 160 nM for human μ-opioid receptor, $> 10{,}000$ nM for human δ-opioid receptor, and 640 ± 87 nM for human κ-opioid receptor (Table 1). [Phe$^1\Psi$(CH$_2$-NH)Gly^2]nociceptin-(1-13)-NH$_2$, which was recently reported to be a peptidyl antagonist of the ORL1 receptor, also showed high affinity for ORL1 (K_i; 1.2 ± 0.12 nM) but low affinity for other opioid receptors (K_i; > 1 μM for all other opioid receptors) (Table 1). The physiological κ_3-opioid receptor agonist naloxone benzoylhydrazone, which was reported to moderate affinity for the KOR-3 receptor, was found to possess moderate affinity for the human ORL1 receptor (K_i; 38 ± 4.4 nM). The binding affinity of this compound to μ-, δ- and κ-opioid receptors was more potent than that

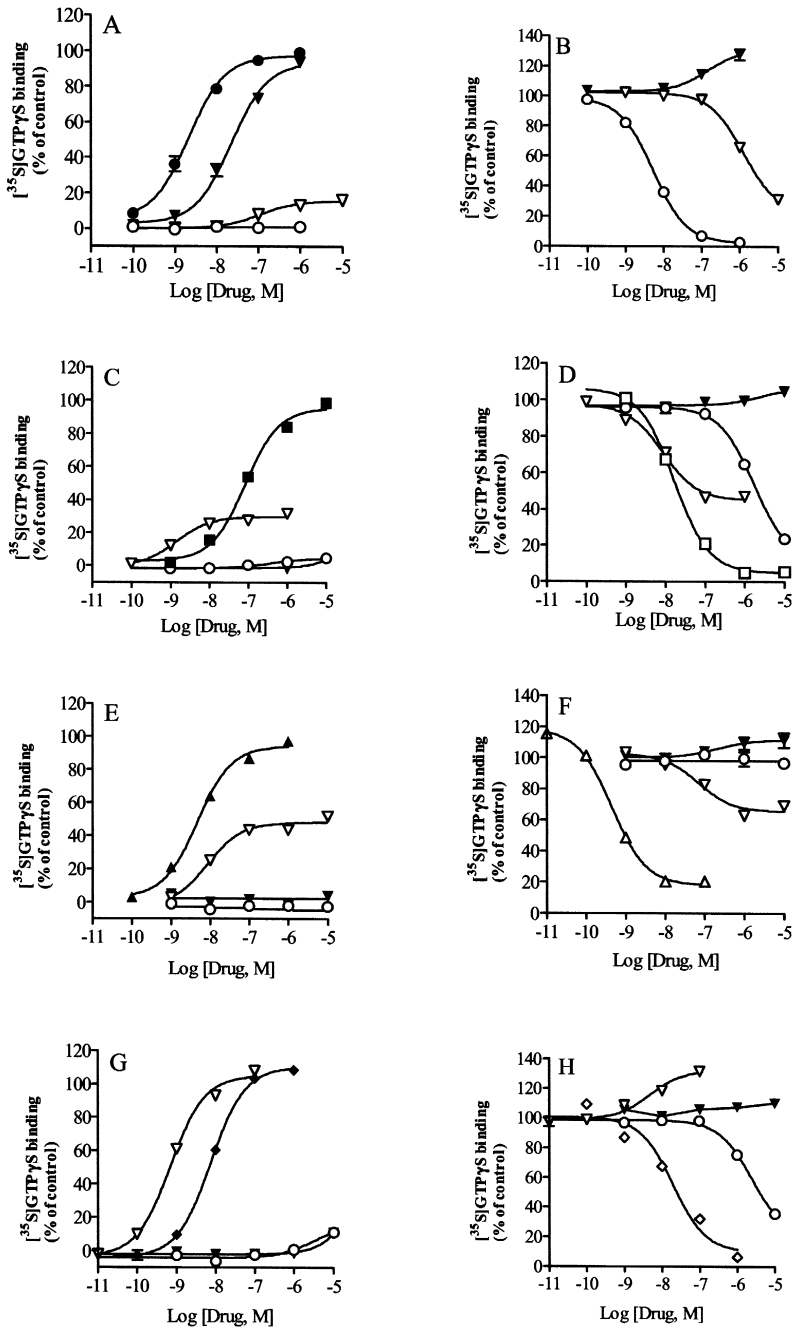

Fig. 2. Effects of J-113397 on basal (A, C, E, G) and agonist-stimulated (B, D, F, H) [^{35}S]GTPγS binding to membranes from CHO cells expressing either ORL1 receptor (A, B), μ-opioid receptor (C, D), δ-opioid receptor (E, F) or κ-opioid receptor (G, H). (●) Nociceptin/orphanin FQ; (○) J-113397; (■) DAMGO; (□) Naloxone; (▲) DADLE; (△) Naltrindole; (◆) U-69593; (◇) norBNI; (▼)Phe^1 Ψ(CH$_2$-NH)Gly$_2$]nociceptin-(1-13)-NH$_2$; (▽) naloxone benzoylhydrazone. Data are mean ± S.E.M. of three experiments.

Table 2
In vitro functional profiles of J-113397 in [^{35}S]GTPγS binding assay

		ORL1[a]	μ[a]	δ[a]	κ[a]
J-113397	IC_{50} (nM)	5.3 ± 0.088	2300 ± 120	> 10,000	4900 ± 320
	EC_{50} (nM)	> 1000	> 10,000	> 10,000	> 10,000
	E_{max} (%)	1.0 ± 0.58	4.8 ± 1.4	−2.6 ± 1.7	13 ± 1.5
NalBzoH[b]	IC_{50} (nM)	2900 ± 88	75 ± 9.4	> 10,000	> 100
	EC_{50} (nM)	110 ± 13	1.7 ± 0.20	12 ± 1.2	0.67 ± 0.12
	E_{max} (%)	16 ± 1.7	32 ± 2.6	54 ± 2.5	110 ± 2.1
[F/G]NC(1–13)[c]	IC_{50} (nM)	> 1000	> 10,000	> 10,000	> 10,000
	EC_{50} (nM)	27 ± 4.6	> 10,000	> 10,000	> 10,000
	E_{max} (%)	94 ± 0.89	4.2 ± 0.88	1.4 ± 3.5	11 ± 1.4
Antagonist	IC_{50} (nM)	–	24 ± 1.0 (Naloxone)	1.0 ± 0.17 (Naltrindole)	27 ± 4.0 (norBNI)
Agonist	EC_{50} (nM)	2.1 ± 0.42	98 ± 16	4.8 ± 0.49	6.2 ± 0.69
	E_{max} (%)	99 ± 1.0 (NC/OFQ)	99 ± 2.1 (DAMGO)	97 ± 0.42 (DADLE)	110 ± 2.1 (U-69593)

[a] Data are mean ± S.E.M. of three experiments.
[b] Naloxone benzoylhydrazone.
[c] [Phe$^1\Psi$(CH$_2$-NH)Gly^2]nociceptin-(1-13)-NH$_2$.

to the ORL1 receptor (K_i; 1.5 ± 0.13 nM for μ-opioid receptor, 11 ± 0.13 nM for δ-opioid receptor, 0.35 ± 0.018 nM for κ-opioid receptor) (Table 1).

Thus, J-113397 is a potent and selective nonpeptidyl ligand for the ORL1 receptor.

3.2. In vitro functional profile of J-113397

In order to assess whether J-113397 possesses the properties of an agonist or antagonist, we examined the effects of J-113397 on [^{35}S]GTPγS binding to the ORL1 receptor and other opioid receptors. Nociceptin/orphanin FQ dose-dependently increased [^{35}S]GTPγS binding to CHO-ORL1 membranes with an EC_{50} value of 2.1 ± 0.42 nM. [Phe$^1\Psi$(CH$_2$-NH)Gly^2]nociceptin-(1-13)-NH$_2$ also increased [^{35}S]GTPγS binding to the same extent as nociceptin/orphanin FQ with an EC_{50} value of 27 ± 4.6 nM. In contrast, J-113397 alone had no effect on basal [^{35}S]GTPγS binding at concentrations up to 1 μM, but completely inhibited nociceptin/orphanin FQ-stimulated [^{35}S]GTPγS binding with an IC_{50} value of 5.3 ± 0.088 nM (Fig. 2A and B, Table 2).

In CHO-μ membranes, naloxone inhibited the DAMGO-stimulated increase in [^{35}S]GTPγS binding with

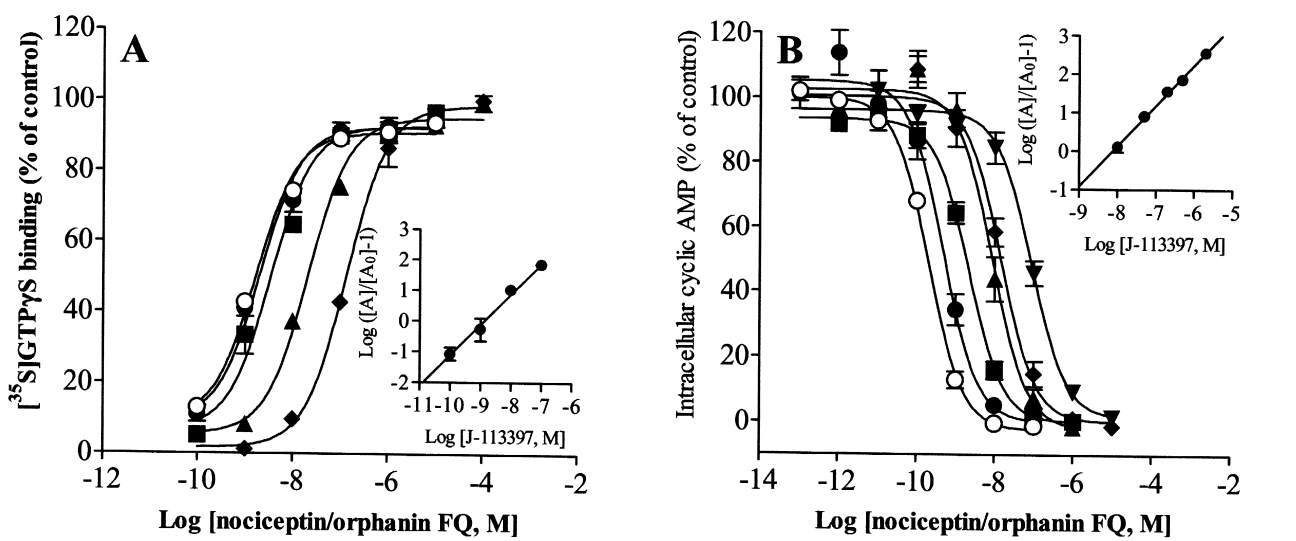

Fig. 3. (A) Antagonistic effects of J-113397 on nociceptin/orphanin FQ-stimulated [^{35}S]GTPγS binding. Radioligand binding was measured at the indicated concentrations of nociceptin/orphanin FQ in the absence (○) or presence of 0.1 (●), 1 (■), 10 (▲) and 100 (♦) nM J-113397. (B) Concentration–response curves for nociceptin/orphanin FQ on forskolin-stimulated accumulation of cyclic AMP in CHO-ORL1 cells in the absence (○) or presence of 10 (●), 50 (■), 200 (▲), 500 (♦) and 200 (▼) nM J-113397. Data are mean ± S.E.M. of three experiments. Inset: Schild plot of J-113397 antagonism.

a high affinity (IC_{50}; 24 ± 1.0 nM), whereas J-113397 inhibited the binding with a low affinity (IC_{50}; 2300 ± 120 nM) (Fig. 2D, Table 2). The opioid κ-receptor agonist U-69593 stimulated [^{35}S]GTPγS binding to CHO-κ membranes (Fig. 2G), which was potently inhibited by a κ-receptor antagonist, nor-BNI (IC_{50}; 27 ± 4.0 nM); in contrast, inhibition by J-113397 was negligible (IC_{50}; 4900 ± 320 nM) (Fig. 2H, Table 2). J-113397 had no effect on basal [^{35}S]GTPγS binding levels in μ-, δ- and κ-membranes and on the DADLE-stimulated [^{35}S]GTPγS binding to δ-membranes at concantrations up to 10 μM (Fig. 2C–G). Thus, these results indicate that J-113397 is a potent and selective antagonist of the ORL1 receptor.

3.3. Manner of inhibition of ORL1 receptor by J-113397

To determine whether J-113397 competitively inhibits nociceptin/orphanin FQ binding to ORL1, concentration–response curves for nociceptin/orphanin FQ in [^{35}S]GTPγS binding and cyclic AMP accumulation were analyzed in the presence or absence of various concentrations of J-113397. The addition of increasing concentrations of J-113397 caused a progressive shift of the nociceptin/orphanin FQ concentration–response curve to the right without any effect on maximal response in the [^{35}S]GTPγS binding study. A Schild plot yielded a pA_2 value of 8.9 ± 0.14 and a slope factor of 1.01 ± 0.075 (Fig. 3A). Nociceptin/orphanin FQ dose-dependently suppressed forskolin-stimulated accumulation of cyclic AMP in CHO-ORL1 cells with an EC_{50} value of 0.22 ± 0.011 nM. Increasing concentration of J-113397 shifted the concentration–response curve for nociceptin/orphanin FQ to the right. From the corresponding Schild plot, the pA_2 value for the compound was calculated to be 8.2 ± 0.14 and the slope factor, to be 1.05 ± 0.052 in cyclic AMP assay (Fig. 3B). These data indicate that J-113397 inhibits the nociceptin/orphanin FQ-ORL1 interaction in a competitive manner.

3.4. Binding affinity of J-113397 for mouse ORL1 receptor

To assess the affinity of J-113397 for mouse ORL1 receptor, we conducted a competition binding study using mouse brains. [^{125}I][Tyr^{14}]nociceptin binding to membrane preparations of mouse brain was completely displaced by J-113397 and nociceptin/orphanin FQ with K_i values of 1.1 ± 0.24 and 0.26 ± 0.027 nM, respectively (Fig. 4A). The affinity of J-113397 was comparable to that for the human receptor. As shown in Fig. 4B, in coronal sections of mouse brain, [^{125}I][Tyr^{14}]nociceptin binding was abundant in the cerebral cortex, hippocampus, amygdala and thalamic/hypothalamic regions. The inhibitory effects of J-113397 were dose-dependent, and more than 50% of the radioligand binding was displaced with 10 nM J-113397.

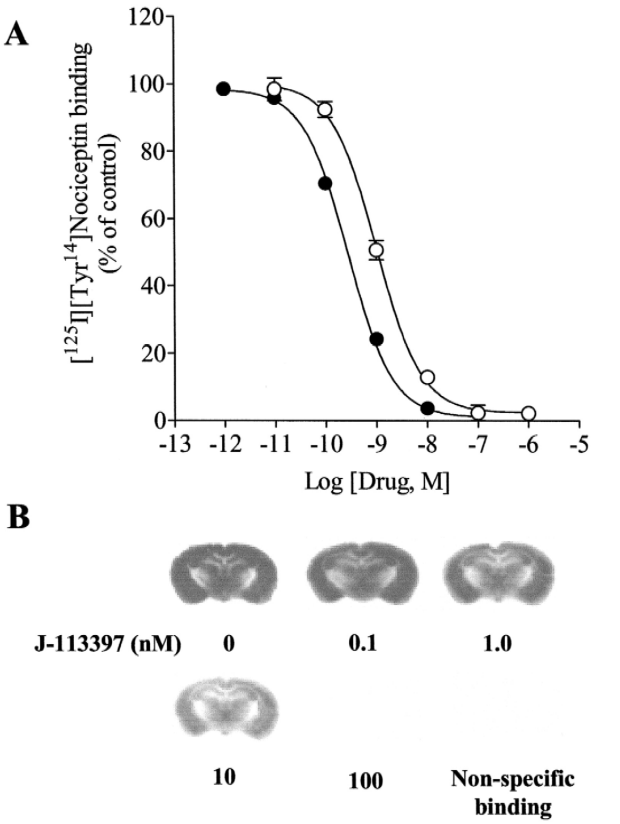

Fig. 4. (A) Effects of nociceptin/orphanin FQ (●) and J-113397 (○) on [^{125}I][Tyr^{14}]nociceptin binding to membrane preparations of mouse brains. The membranes were incubated with 50 pM [^{125}I][Tyr^{14}]nociceptin. Data are mean ± S.E.M. of three experiments. (B) Effects of J-113397 on [^{125}I][Tyr^{14}]nociceptin autoradiography. Coronal sections of mouse brains at the level of the thalamus were incubated with the radioligand as described in Section 2.

Thus, J-113397 possesses high affinity for the mouse ORL1 receptor as well as the human receptor.

4. In vivo antagonistic effects of J-113397

To examine in vivo antagonism by J-113397, we evaluated the effects of J-113397 in a tail-flick test. Mice were given an i.c.v. injection of saline or nociceptin/orphanin FQ (0.01–1 nmol). As shown in Fig. 5A, no obvious change in tail-flick latency was found after i.c.v. injection of saline. Nociceptin/orphanin FQ shortened the latency at doses of more than 0.1 nmol. The effect of nociceptin/orphanin FQ lasted more than 60 min at the high concentration, with a maximal decrease at 15 min after agonist injection. J-113397 dose-dependently inhibited nociceptin/orphanin FQ-induced shortening of the tail-flick latency of mice and 30 mg/kg of J-113397 completely reversed hyperalgesia elicited by nociceptin/orphanin FQ (Fig. 5B). However, J-113397 alone did not exert significant effects on baseline tail-flick latency (data not shown).

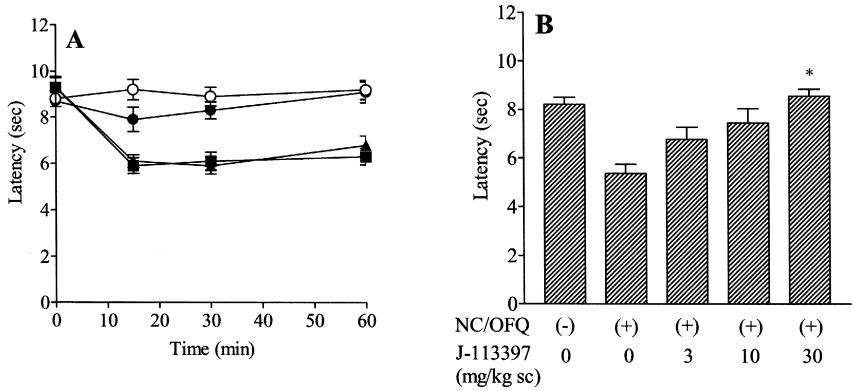

Fig. 5. (A) Time course of the effects of i.c.v. administered nociceptin/orphanin FQ in the tail-flick test. Data are mean ± S.E.M. of eight experiments. (○) Saline; (●) 0.01 nmol; (■) 0.1 nmol; (▲) 1 nmol nociceptin/orphanin FQ. $P < 0.05$ as compared with saline. (B) Antagonistic effects of J-113397 on nociceptin/orphanin FQ (0.1 nmol, i.c.v.)-induced hyperalgesia in the tail-flick test. Mice were subcutaneously administered 3–30 mg/kg J-113397 prior to nociceptin/orphanin FQ. Tail-flick latency was measured 15 min after the administration of nociceptin/orphanin FQ ($n = 8$). $P < 0.05$ as compared with saline. NC/OFQ; nociceptin/orphanin FQ.

These data indicate that J-113397 is an effective antagonist in vivo as well as in vitro.

5. Discussion

We have described the in vitro biological profiles of J-113397, the most potent and selective nonpeptidyl ORL1 antagonist yet reported. Competition binding study indicated that J-113397 possesses high affinity and more than 360-fold selectivity for the ORL1 receptor over other opioid receptors. [^{35}S]GTPγS binding study and cyclic AMP assay revealed that J-113397 is a potent and competitive antagonist of ORL1, which possesses a weak antagonist activity on μ- and κ-opioid receptors only at a high concentration. Therefore, J-113397 is a potent and competitive ORL1 receptor antagonist without any agonistic effects on other opioid receptors.

The nociceptin/orphanin FQ derivative [Phe^{1}Ψ(CH$_2$-NH)Gly^2]nociceptin-(1-13)-NH$_2$ has recently been reported to behave as a selective antagonist of the ORL1 receptor in guinea pig ileum and rat vas deferens (Guerrini et al., 1998). However, this peptide was reported to be a full agonist in a cyclic AMP assay of CHO cells transfected with human ORL1 (Butour et al., 1998; Kapusta et al., 1999) and a partial agonist/antagonist in both a [^{35}S]GTPγS binding assay and a cyclic AMP assay of mouse N1E-115 neuroblastoma (Olianas et al., 1999). Our present study using CHO-ORL1 cells demonstrated that [Phe^{1}Ψ(CH$_2$-NH)Gly^2]nociceptin-(1-13)-NH$_2$ acts as a full agonist for ORL1 in [^{35}S]GTPγS binding assay. In vivo, central administration of the peptide was reported to inhibit morphine analgesia in mice (Grisel et al., 1998) and suppress the nociceptive flexor reflex in rats (Xu et al., 1998), which are agonist properties, while peripheral administration inhibited peripheral nociceptin/orphanin FQ-induced hypotension and bradycardia (Madeddu et al., 1999). Therefore, it is still debatable whether [Phe^{1}Ψ-(CH$_2$-NH)Gly^2]nociceptin-(1-13)-NH$_2$ acts as an antagonist or agonist of the ORL1 receptor.

Naloxone benzoylhydrazone, originally reported as a mouse κ$_3$-opioid receptor agonist (Gistrak et al., 1989), was recently found to possess a moderate affinity for KOR-3, the mouse homologue of the ORL1 receptor (Pan et al., 1995, 1996). Our present results also demonstrated that naloxone benzoylhydrazone possesses a moderate affinity for the human ORL1 receptor and human δ-opioid receptor, and a high affinity for human μ- and κ-opioid receptors. In the [^{35}S]GTPγS study, naloxone benzoylhydrazone completely inhibited nociceptin/orphanin FQ-stimulated [^{35}S]GTPγS binding in CHO-ORL1 with a low affinity (IC$_{50}$; 2900 ± 88 nM) (Fig. 2B, Table 2), but partially inhibited DAMGO-stimulated [^{35}S]GTPγS binding, and partially increased basal [^{35}S]GTPγS binding by itself in CHO-μ membranes (Fig. 2C and D, Table 2). This compound also showed partial agonist/antagonist activity on δ-opioid receptors (Fig. 2E and F, Table 2). Furthermore, naloxone benzoylhydrazone alone potently increased basal [^{35}S]GTPγS binding in CHO-κ membranes with an EC$_{50}$ value of 0.67 ± 0.12 nM (Fig. 2G). Thus, naloxone benzoylhydrazone is a potent κ$_3$-opioid receptor agonist, a potent κ (κ$_1$)-opioid receptor agonist, a partial agonist of the μ-opioid receptor, and a moderate ORL1 receptor antagonist. Although naloxone benzoylhydrazone possesses moderate affinity for the ORL1 receptor, low selectivity of the compound might make its use for in vivo characterization of the ORL1 receptor difficult.

In the present study, J-113397 was found to possess high affinity for mouse the ORL1 receptor as well as the human receptor. In vivo, i.c.v. injection of NC/OFQ at a concentration of more than 0.1 nmol significantly shortened the tail-flick latency in mice, which was similar to the findings of Reinscheid et al. (1995). J-113397 (s.c.) dose-dependently inhibited nociceptin/orphanin FQ (i.c.v.)-in-

duced hyperalgesia in the tail-flick assay without altering baseline tail-flick latency. These results indicate that J-113397 possesses in vivo as well as in vitro antagonistic activity against the ORL1 receptor. Therefore, J-113397 is the most potent and selective ORL1 receptor antagonist, and should be useful for characterization of the ORL1 receptor in vivo.

In summary, J-113397 is a potent and selective nonpeptidyl ORL1 receptor antagonist without agonistic effects on other opioid receptors. In vivo, J-113397 (s.c.) antagonized nociceptin/orphanin FQ-induced responses and therefore should be an excellent pharmacological tool to clarify the physiological roles of nociceptin/orphanin FQ and ORL1, and to explore the therapeutic potential of ORL1 receptor antagonists.

Acknowledgements

The authors would like to thank Dr. S. Nishimura, Dr. M. Yoshida and Dr. H. Morishima for their useful discussions and Ms. A. Dobbins for her critical reading of the manuscript.

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