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Pharmacological profiles of a novel opioid receptor-like1 (ORL_1) receptor antagonist, JTC-801

*,[1]Hideki Yamada, [1]Hiromitsu Nakamoto, [1]Yasunori Suzuki, [1]Takao Ito & [1]Kazuo Aisaka

[1]Central Pharmacological Research Institute, JT Inc., 1-1 Murasaki-cho, Takatsuki, Osaka, 569-1125, Japan

1 Pharmacological effects of a novel opioid receptor-like1 (ORL_1) receptor antagonist, [N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl) benzamide monohydrochloride] (JTC-801), were examined in *in vitro* and *in vivo*.

2 JTC-801 inhibited the binding of [^{3}H]-nociceptin to human ORL_1 receptors expressed in HeLa cells with a K_i value of 44.5 nM.

3 JTC-801 completely antagonized the suppression of nociceptin on forskolin-induced accumulation of cyclic AMP (IC_{50}: 2.58 μM) using ORL_1 receptor expressing HeLa cells *in vitro*.

4 In *in vivo*, when given intravenously at dosages of 0.01 mg kg^{-1} and above, or orally at dosages 1 mg kg^{-1} and above, JTC-801 antagonized the nociceptin-induced allodynia in mice.

5 Effects of JTC-801 on various nociceptive models were examined. In mouse hot-plate test, JTC-801 prolonged escape response latency (ERL) to exposed heat stimulus with minimum effective doses (MED) of 0.01 mg kg^{-1} by i.v. or 1 mg kg^{-1} by p.o.

6 In the rat formalin test, JTC-801 reduced both the first and second phases of the nociceptive response with MED of 0.01 mg kg^{-1} by i.v. administration or 1 mg kg^{-1} by p.o. administration. This anti-nociceptive action of JTC-801 was not inhibited by naloxone (10 mg kg^{-1}, s.c.).

7 We have demonstrated that JTC-801 antagonizes the ORL_1 receptor response, and that JTC-801 has efficacious and potent anti-nociceptive effects in acute pain animal models not only by intravenous injection but also oral administration. These results suggest that JTC-801 may represent a new class of analgesics.

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Keywords: Nociceptin; ORL_1; JTC-801; nociceptin receptor antagonist; morphine; allodynia; hot-plate test; formalin test

Abbreviations: ERL, escape response latency; MED, minimum effective doses; ORL_1, opioid receptor-like protein

Introduction

Nociceptin, also referred to as orphanin FQ (hereafter nociceptin), has been identified as an endogenous ligand of opioid receptor-like (ORL_1) receptor (Meunier *et al*., 1995; Reinscheid *et al*., 1995). ORL_1 receptor mRNA is densely expressed in the cerebral cortex, thalamus, subfornical organ, habenulae, hypothalamus, central grey, dorsal raphe, locus coeruleus, and also in the dorsal horn of the spinal cord and dorsal root ganglia (Bunzow *et al*., 1994; Wick *et al*., 1994). Thus, ORL_1 receptors may be involved in a wide variety of physiological functions. Nociceptin is 17 amino acids long and has some homology with the dynorphin family of peptides, but has little affinity for traditional opioid receptors such as δ, κ, μ. Nociceptin has been shown to produce several effects, such as pain modulation, motor modulation, suppression of spatial learning and stimulation of food intake (Meunier, 1997; 2000; Calo' *et al*., 2000). Recently, a mutant of nociceptin, [Phe$^1\psi$(CH_2-NH)Gly^2]NC(1–13)-NH_2, was reported to be a selective antagonist of ORL_1 receptor in guinea-pig ileum and mouse vas deferens (Guerrini *et al*., 1998). On the other hand, this pseudopeptide was described as an agonist in controlling cyclic AMP levels in CHO cells transfected with human ORL_1 receptor (Butour *et al*., 1998; Kapusta *et al*., 1999). Thus, the role of nociceptin in *in vivo* pain signalling remains unclear due to the lack of a selective ORL_1 receptor antagonist.

We successfully synthesized a novel and oral active ORL_1 receptor antagonist, JTC-801, [N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl) benzamide monohydrochloride] (Shinkai *et al*., 2000). In the present study, we describe the pharmacological characterization of JTC-801 *in vitro* and *in vivo*. We examined the specificity and antagonism of JTC-801 on ORL_1 receptor *in vitro*, and also the effects of JTC-801 on various nociceptive animal models *in vivo*.

Methods

Drugs and chemicals

JTC-801 (molecular weight: 447.96) was synthesized by the JT Central Pharmacological Research Institute (Osaka, Japan). Nociceptin was purchased from the Peptide Institute (Osaka, Japan). [^{3}H]-nociceptin (163 Ci mmol^{-1}) and cyclic AMP EIA system were from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). DAMGO, [Tyrosyl-3,5-^{3}H(N)]-([^{3}H]-DAMGO), was obtained from NEN Life Science Products (Boston, U.S.A.). Tris(hydroxymethyl)aminomethane (Tris), (p-amidoinophenyl) methanesulphonyl fluoride hydrochloride, $MgCl_2$, 3-isobutyl-1-methylxanthine, and

*Author for correspondence; E-mail: hideki.yamada@ims.jti.co.jp

D-sorbitol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), ethylene glycol bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), dimethyl sulphoxide, and methylcellulose were from Nacalai Tesque (Kyoto, Japan). Bovine serum albumin, polyethyleneimine, naloxone hydrochloride, and forskolin were from Sigma-Aldrich Japan (Tokyo, Japan); cell culture medium (Eagle's MEM) was from Nikkenn Bio Medical Laboratory (Kyoto, Japan); foetal calf serum (FCS) was from Intergen (NY, U.S.A.); penicillin-streptomycin was from Gibco BRL (Tokyo, Japan); and morphine hydrochloride (molecular weight: 366.27) was from Takeda Chemical Industries (Osaka, Japan).

In vitro *study*

Human ORL_1 receptor binding affinity Human ORL_1 receptor expressed in HeLa cells, which were transfected by JT Central Pharmacological Research Institute, were harvested and homogenized in 50 mM Tris buffer (pH 7.4) containing 1 mM EDTA. After centrifugation for 30 min at $40{,}000 \times g$, the pellets were resuspended in buffer containing 50 mM Tris, supplemented with 10 mM $MgCl_2$ and 2 mM EGTA, and used as membrane preparations. Fifty mM Tris (pH 7.4) supplemented with 2 mM EDTA and 0.1 mM (p-amidoinophenyl) methanesulphonyl fluoride hydrochloride containing 0.2% bovine serum albumin was used for the binding buffer. For saturation binding assay, the cell membrane preparations were incubated for 60 min at 24°C with various concentrations of [^{3}H]-nociceptin. Nonspecific binding was determined in the presence of 1 μM unlabelled nociceptin. For competitive assay, the cell membrane preparations (4.17 μg well^{-1}) were incubated for 60 min at 24°C with 50 pM [^{3}H]-nociceptin in the presence of various concentrations of JTC-801 (10 nM – 10 μM). JTC-801 was dissolved in dimethyl sulphoxide and diluted in binding buffer, and then added to the incubation mixture. Final concentration of vehicle was 1% dimethyl sulphoxide in binding buffer. This concentration of dimethyl sulphoxide did not affect the binding of [^{3}H]-nociceptin. After incubation for 60 min, the membrane preparations were rapidly filtrated over Whatman GF/B glass filters which were pretreated with 0.1% polyethyleneimine, and the radioactivity on each filter was measured by liquid scintillation counting (Topcount; A9912V, Packard, U.S.A.). IC_{50} values were calculated as the concentration of JTC-801 required to displace 50% inhibition of the [^{3}H]-nociceptin. The results of binding assays are presented as the mean ± s.e.mean for three separate experiments.

Human opioid receptors binding affinity The binding affinities for human δ-, κ-, and μ-opioid receptors were determined with [^{3}H]-naltrindole for δ-opioid receptor and with [^{3}H]-diprenorphine for κ-, and μ-opioid receptors, and were performed by Panlabs Taiwan, Ltd. Pharmacology laboratories. Each membrane fraction of CHO cells expressing human δ-, κ-, and μ-opioid receptor was incubated in 50 mM Tris (pH 7.4) with radioligand at 25°C for 2 h (δ-opioid) or 1 h (κ- and μ-opioid). Nonspecific binding was determined in the presence of 10 μM naloxone. IC_{50} values were calculated as the concentration of JTC-801 required to displace 50% inhibition of each ligand.

Rat ORL_1 receptor and μ-opioid receptor binding affinity The binding affinities for rat cerebrocortical ORL_1 and μ-opioid receptors were determined with [^{3}H]-nociceptin and [^{3}H]-DAMGO, respectively. Male Sprague Dawley (SD) rats were decapitated following cervical dislocation. The rat cerebral cortex was then dissected rapidly and homogenized on ice with Tris buffer (pH 7.4). After incubation at 37°C for 30 min, the homogenate was centrifuged at $30{,}000 \times g$ for 20 min at 4°C and the pellet resuspended in Tris buffer. This procedure was repeated twice. Finally, 250 and 377.5 μg of membrane protein from rat cerebral cortex was used for ORL_1 and μ-opioid receptor binding assays, respectively. Nonspecific binding was determined in the presence of 1 μM nociceptin or 10 μM naloxone. IC_{50} values were calculated as the concentration of JTC-801 required to displace 50% inhibition of each ligand.

Antagonism for ORL_1 receptor HeLa cells expressing human ORL_1 receptor were seeded onto 24-well culture plates (Nippon Becton Dickinson; Tokyo, Japan) at a density of 10^5 cells well^{-1} and cultured in Eagle's MEM containing 10% FCS supplemented with 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. After cultivation for 24 h, the incubation medium was exchanged to 1 ml of Eagle's MEM containing 0.1% bovine serum albumin and incubated for 2 h at 37°C. Then, stimulation was started by exchanging the incubation medium for reaction mixture containing 10 μM forskolin, 2 mM 3-isobutyl-1-methylxanthine and various concentrations of JTC-801 (0.1 – 10 μM) in the presence or absence of various concentrations of nociceptin (0.001 – 10 pM). JTC-801 was dissolved in dimethyl sulphoxide, diluted in incubation buffer, then added to the reaction mixture. Final concentration of vehicle was 1.2% dimethyl sulphoxide in the incubation buffer. After incubation for 15 min, the reaction mixture was removed and the cells frozen in MeOH containing dry ice to terminate the stimulation. The concentration of cyclic AMP was measured using a commercial EIA kit. The data of cyclic AMP accumulation are represented as per cent of control. IC_{50} values were calculated as the concentrations of ligand producing 50% of the maximal inhibition in cyclic AMP accumulation. The values of IC_{50} are presented as the mean ± s.e.mean for six separate experiments.

In vivo *study*

Animals These experiments were performed in accordance with the guidelines for animal experimentation set by the ethics committee for animal use at Japan Tobacco. Male ICR (CD-1) mice (3 weeks old) or male SD rats (6 weeks old) were purchased from Charles River Japan (Yokohama, Japan). Animals were housed at 10 (mice) or 3 (rats) per cage in a room controlled for temperature (23.0 ± 3.0°C), humidity (55 ± 15%) and light (0800 – 2000 h), and maintained with a standard laboratory chow diet (CRF-1, Oriental Yeast; Ibaraki, Japan) and water *ad labium*. Animals were used in the study at 4 (mice) or 7 (rats) weeks old, after more than 1-week acclimation of the housing environment.

Nociceptin-induced allodynia test

Studies on allodynia were carried out according to the method of Okuda-Ashitaka *et al.* (1996) with minor modifications. JTC-801 was dissolved in 5% sorbitol, and injected into the tail vein (0.003–0.1 mg kg^{-1}) 5 min before the injection of nociceptin, or suspended in 0.5% methylcellulose and administered orally (0.3–10 mg kg^{-1}) 60 min before the nociceptin injection. Nociceptin (50 pg 5 μl^{-1} $body^{-1}$) was injected into the subarachoid space of conscious mice by the method of Hylden & Wolcox (1980). After the injection of nociceptin, each mouse was placed in an individual 15 × 24 × 12 cm Plexiglas enclosure. Allodynia was assessed once every 5 min for 20 min by light stroking of the flank with a paintbrush by an experienced observer blind to the drug condition. The allodynic responses were ranked as follows: 0, no response; 1, mild squeaking with attempts to move away from the stroking probe; 2, vigorous squeaking evoked by the stroking probe and strong efforts to escape. The effect of the test compound was evaluated on the basis of total allodynia scores over the 20 min from each group.

Hot-plate test

Each mouse was placed on a hot plate (7250, Ugo Basile, Italy), maintained at 55.5±0.5°C, and the time elapsed until licking of its hind limbs or jumping to escape was defined as escape response latency (ERL) and used as an indicator of the pain. The ERL was measured to the nearest one second by an experienced observer blind to the drug condition. In previous studies, we observed that animals with an ERL exceeding 15 s prior to drug administration, tended to have large deviations in values for later evaluations. Therefore, we decided to exclude those animals, for the drug evaluations. Mice with an ERL of 30 s or less at first measurement, and mice with an ERL of 15 s or less at second measurement were selected prior to the experiment. Values from the next two measurements, with an interval of 30 min, were averaged to give the basal ERL, which was usually in the range of 2–5 s. A cut-off latency of 30 ms was imposed to prevent possible foot tissue damage. JTC-801 was dissolved in 5% sorbitol and injected into the tail vein (0.003–0.1 mg kg^{-1}) or suspended in 0.5% methylcellulose and administered orally (0.3–10 mg kg^{-1}). Fifteen, 30, 45, and 60 min after the intravenous administration or 30, 60, 90, 120, and 180 min after the oral administration the ERL was determined. In a separate experiment, the effects of morphine dissolved in physiological saline (0.1–3 mg kg^{-1}, i.v.) or in distilled water (1–30 mg kg^{-1}, p.o.) were examined in the same manner.

Formalin test

Limb licking response was induced by subcutaneous injection of 50 μl of 5% formalin to the left hind limb of each rat. The first 5 min (from immediately after the injection of formalin) and the subsequent 15 min (15–30 min post-injection) were designated as the first and second phases, respectively. The limb licking time during each of the phases was determined and used as an indicator of pain. JTC-801 was dissolved in 5% sorbitol and injected into the tail vein (0.003–0.1 mg kg^{-1}) 5 min before formalin injection, or suspended in 0.5% methylcellulose and administered orally (0.3–10 mg kg^{-1}) 60 min before formalin injection. In a separate experiment, the effects of morphine dissolved in physiological saline (0.1–3 mg kg^{-1}, i.v.) or in distilled water (1–30 mg kg^{-1}, p.o.) were examined in the same manner.

Table 1 Inhibition of [^{3}H]-nociceptin binding by JTC-801 in human ORL_1 receptor

JTC-801 (nM)	K_d (pM)	B_{max} (pmol mg $protein^{-1}$)
0	32.7±4.2	2.8±0.3
50	43.1±6.4	2.1±0.1
100	55.0±1.4	1.6±0.1
150	97.1±7.7	1.6±0.1
K_i (nM)	44.5±21.6	

Saturation study was performed with [^{3}H-nociceptin (30–300 pM in the membrane preparation of HeLa cell expressing human ORL_1 receptor. K_i value was calculated from Dixon analysis. Each value was the mean±s.e.mean from three independent experiments.

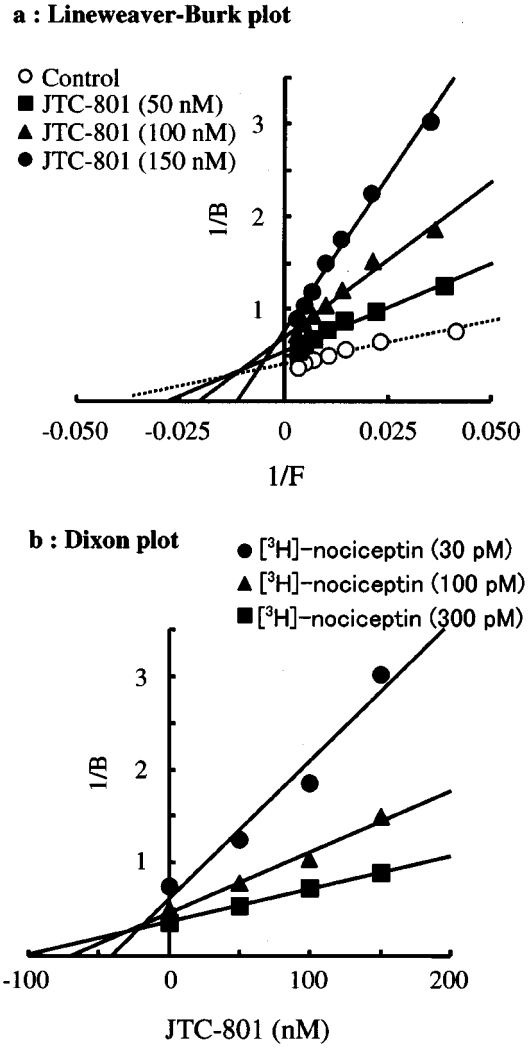

Figure 1 Mode of inhibition of JTC-801 on human ORL_1 receptor. Saturation study was performed with [^{3}H]-nociceptin (30–300 pM) in the membrane preparation of HeLa cells expressing human ORL_1 receptor. Binding reaction was performed at room temperature for 1 h. (a) Upper panel shows the Lineweaver-Burk plot. (b) Lower panel shows the Dixon plot. A representative result from three independent experiments is shown.

Naloxone antagonism

The antagonistic effect of naloxone, a non-specific opioid antagonist, on the anti-nociceptive effect of JTC-801 and morphine was examined by formalin stimulation test. Limb licking response was induced by subcutaneous injection of 50 μl of 5% formalin to the left hind limb of each rat. The first 5 min (from immediately after the injection of formalin) and the subsequent 15 min (15–30 min post-injection) were designated as the first and second phases, respectively. The limb licking time during each of the phases was measured and used as an indicator of pain. Fifteen min before the injection of formalin, naloxone (10 mg kg^{-1}, dissolved in physiological saline) was given subcutaneously. Five min before the injection of formalin, JTC-801 and morphine were dissolved in 5% sorbitol and given into the tail vein at doses of 0.03 and 1.0 mg kg^{-1}, respectively. JTC-801 (3.0 mg kg^{-1}) and morphine (30 mg kg^{-1}) were administered orally 60 min before the formalin injection.

Table 2 Binding affinity of JTC-801 for ORL_1, and for δ, κ, μ opioid receptors

Receptor	*Origin*	*IC_{50} value* (nM)	*Ligand used* (pM)	*Ligand* K_d value (pM)
ORL_1	Human	94	50	24
ORL_1	Rat cerebral cortex	472	500	131
δ	Human	>10000	900	490
κ	Human	>10000	600	400
μ	Human	325	600	410
μ	Rat cerebral cortex	1831	1000	823

Statistics

Allodynia data are presented as the mean ± s.d. of 10 mice. Statistical analysis was performed with Kruskal Wallis test, followed by Steel's test for multiple comparisons with vehicle-control group. The result of hot-plate test and formalin test are presented as the mean ± s.d. of 10–11 mice or 7 rats, respectively. Statistical analysis of hot-plate test and formalin test was performed with one-way analysis of variance (ANOVA), followed by Dunnett's test for multiple compar-

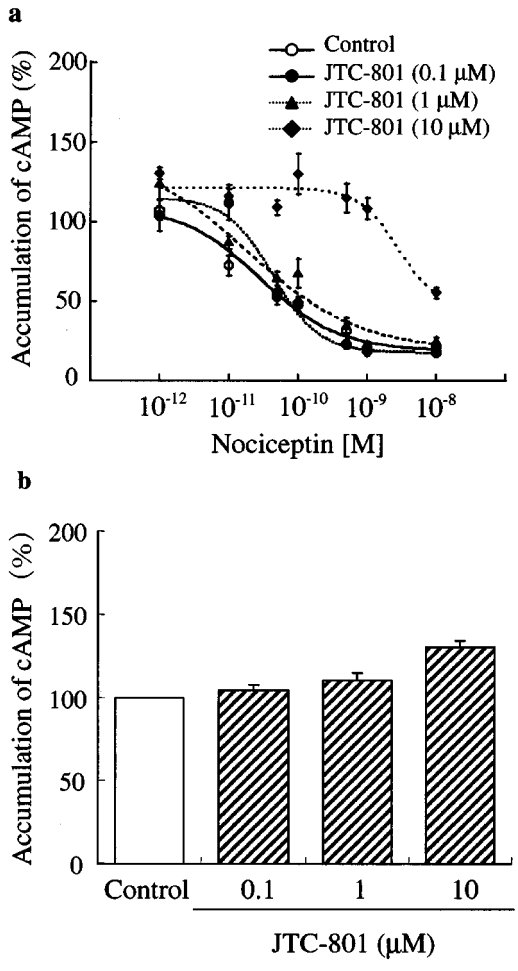

Figure 2 (a) Effect of JTC-801 on nociceptin-produced suppression of cyclic AMP accumulation elicited by forskolin (10 μM) in HeLa cells expressing ORL_1 receptors. (b) Effect of JTC-801 on 10 μM forskolin-produced accumulation of cyclic AMP in HeLa cells expressing ORL_1 receptors. Data are the mean ± s.e.mean of six experiments.

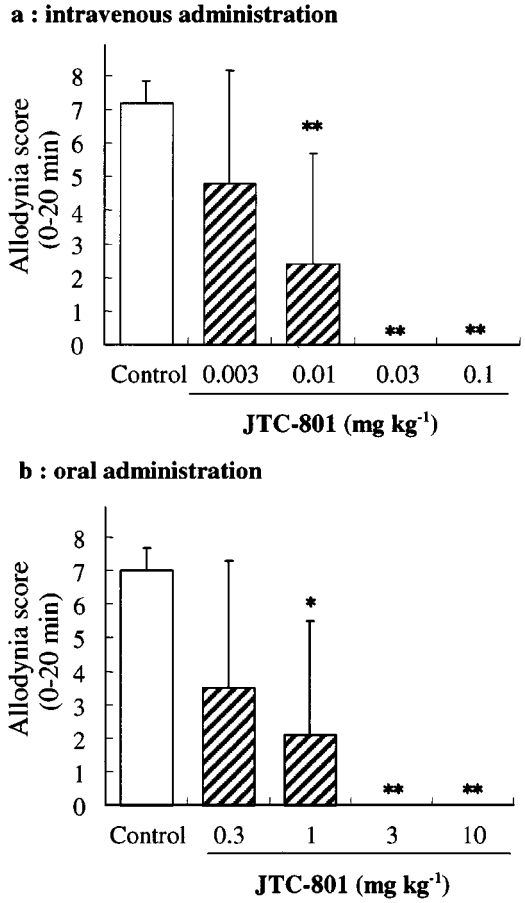

Figure 3 Effect of JTC-801 on nociceptin-induced allodynia. Nociceptin (50 pg 5 μl^{-1} $body^{-1}$) was injected into the subarachnoid space of conscious mice. Assessment of allodynia was made by totaling allodynia score of the first 20 min after the injection of nociceptin at 5 min intervals. JTC-801 was injected into the tail vein 5 min before the injection of nociceptin (a), or administered orally 60 min before the nociceptin injection (b). Values represent mean ± s.d. of 10 mice. $*P<0.05$, $**P<0.01$, significantly different from vehicle treated controls (Kruskal Wallis test followed by Steel's test).

isons with vehicle-control. Naloxone antagonism test was analysed by ANOVA, followed by Tukey-Kramer test for multiple comparisons with each group. $P<0.05$ was considered statistically significant.

Results

Binding affinity of JTC-801 for ORL_1 and opioid receptors

To analyse the kinetic property of JTC-801, Lineweaver-Burk plot was used. JTC-801 inhibited [^{3}H]-nociceptin binding to ORL_1 receptor expressed in HeLa cells. The K_d value increased and B_{max} decreased concentration-dependently with JTC-801 (Table 1, Figure 1a). The results indicate that the kinetic property of JTC-801 with ORL_1 receptor is a mixture of competitive and noncompetitive types. The K_i value of JTC-801 was 44.5±21.6 nM, as analysed by Dixon plot (Table 1, Figure 1b).

A summary of the binding affinity of JTC-801 for ORL_1 and opioid receptors is given in Table 2. JTC-801 inhibited [^{3}H]-nociceptin binding to ORL_1 receptor expressed in HeLa cells with an IC_{50} value of 94±8.6 nM at a [^{3}H]-nociceptin concentration of 50 pM. JTC-801 weakly inhibited the binding of the ligands to human δ receptor ($IC_{50}>10$ μM), κ receptor ($IC_{50}>10$ μM), and μ receptor ($IC_{50}=325$ nM). In rat cerebrocortical membrane, JTC-801 inhibited ORL_1 receptor ($IC_{50}=472$ nM) and μ receptor ($IC_{50}=1831$ nM).

Antagonism of ORL_1 receptor

Nociceptin suppressed the accumulation of cyclic AMP elicited by 10 μM forskolin in a dose related manner. JTC-801 at a concentration of 10 μM reversed the inhibitory action of nociceptin against forskolin-induced increase in cyclic AMP level (IC_{50}: 2.58 μM, 1 nM of nociceptin used) (Figure 2a). In the presence of JTC-801 alone, the production of cyclic AMP was not affected (Figure 2b).

Effects of JTC-801 on nociceptin-induced allodynia

In order to investigate the *in vivo* antagonism of JTC-801, mice allodynia model was used. As shown in Figure 3, i.t.

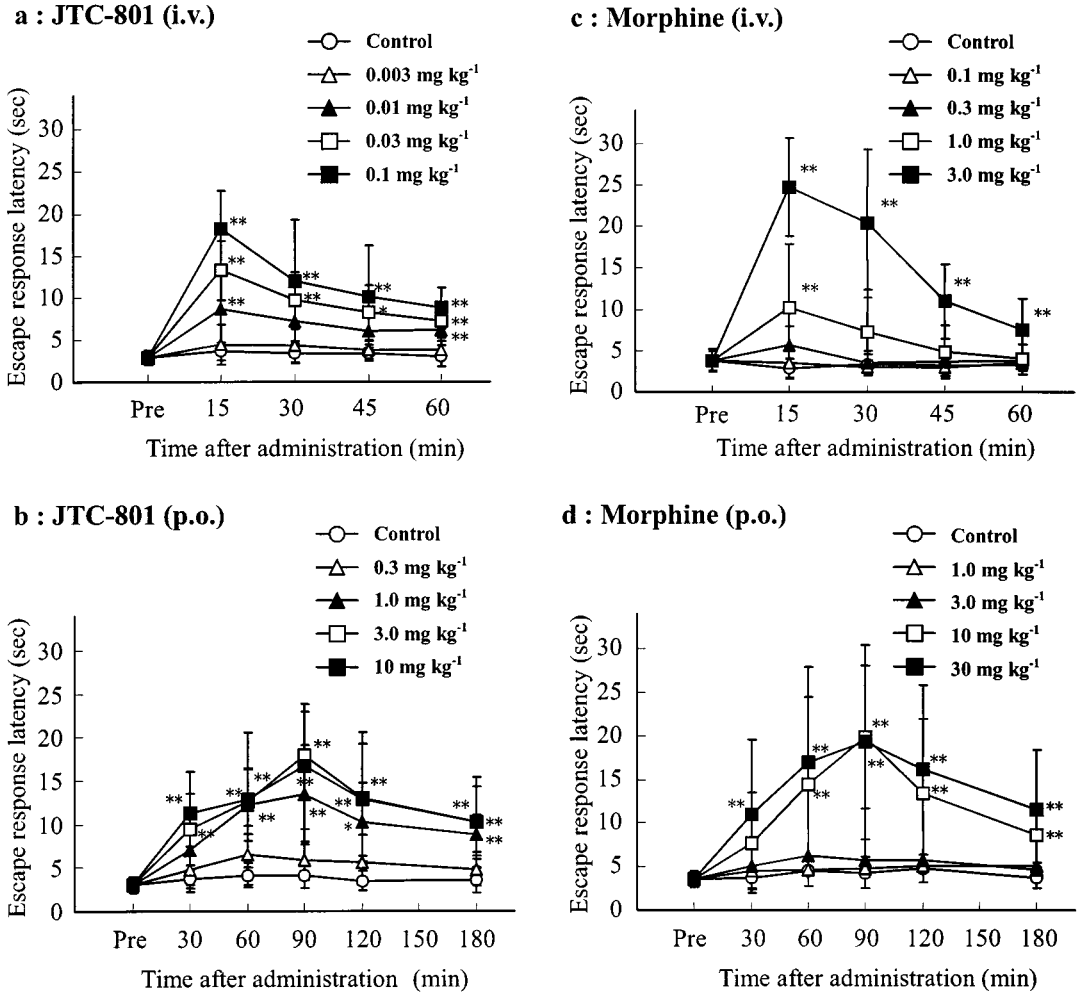

Figure 4 Effect of JTC-801 (a,b) and morphine (c,d) in the mouse hot plate test. Escape response latency (ERL) was determined 15, 30, 45 and 60 min after the i.v. (a,c) or 30, 60, 90, 120 and 180 min after p.o. (b,d) injection of test compounds. Values represent mean±s.d. of 10 or 11 mice. *$P<0.05$, **$P<0.01$, significantly different from vehicle treated controls (one-way ANOVA followed by Dunnett's test).

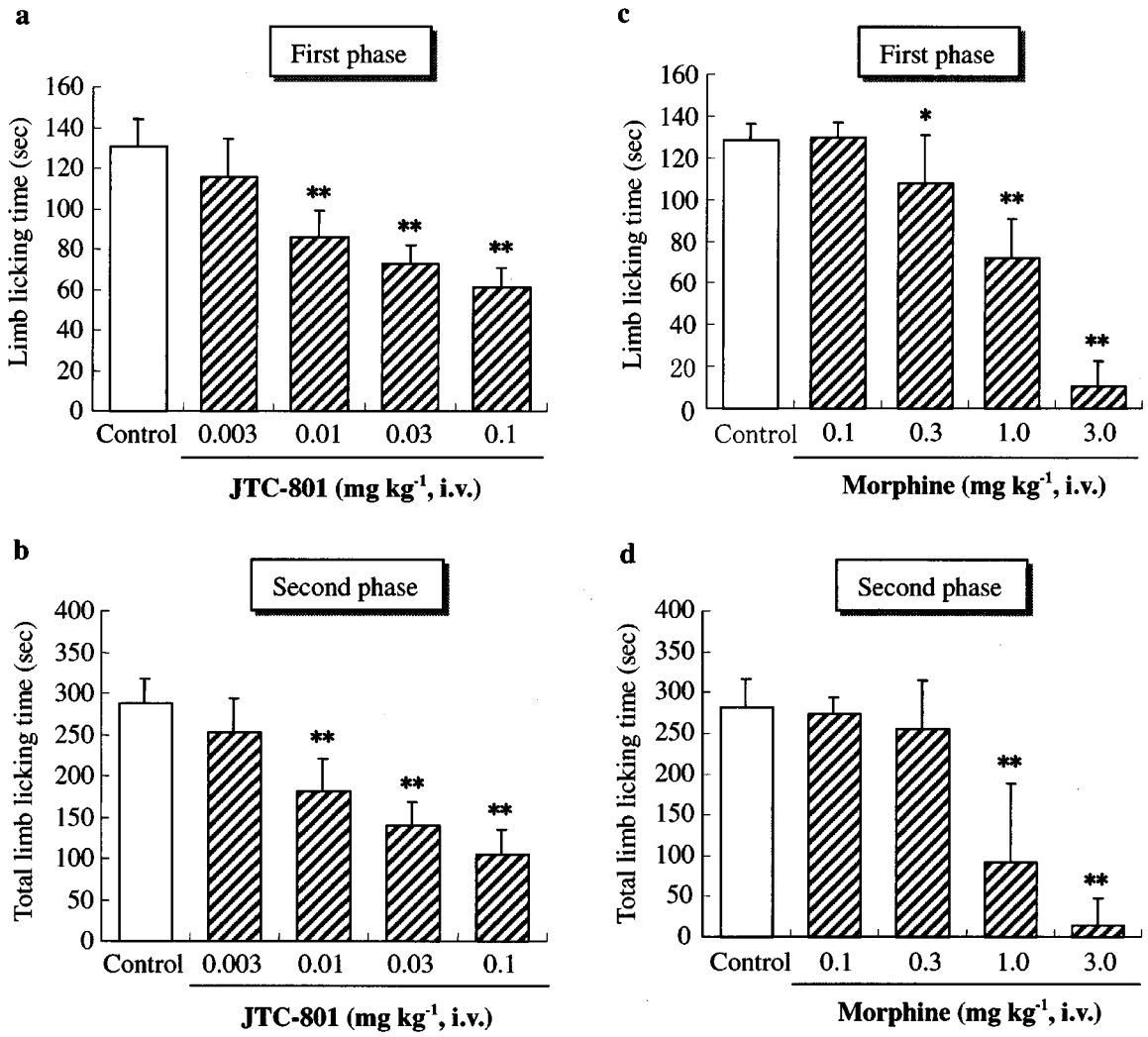

Figure 5 Effect of JTC-801 (a,b) and morphine (c,d) in the rat formalin test. JTC-801 and morphine were administered i.v. 5 min before animals received 50 μl of 5% formalin into the left hind paw. Results are expressed as mean $\pm$ s.d. of time spent licking the injected paw during the first (0–5 min; a,c) and second (15–30 min; b,d) phases of seven rats. $^*P<0.05$, $^{**}P<0.01$, significantly different from vehicle treated controls (one-way ANOVA followed by Dunnett's test).

administration of nociceptin (50 pg 5 μl^{-1} body^{-1}) produced an allodynic response. JTC-801, when given intravenously, inhibited nociceptin-induced allodynia in a dose related manner, with statistical significance at 0.01 mg kg^{-1} and above compared to that of the vehicle group. At doses of 0.03 mg kg^{-1} and above, the onset of allodynia was completely inhibited (Figure 3a). JTC-801, when administered orally at doses of 1 mg kg^{-1} and above, also inhibited nociceptin-induced allodynia in mice. At doses of 3 mg kg^{-1} and above, the onset of allodynia was completely inhibited. (Figure 3b).

Effects of JTC-801 on escape response latency in the mouse hot-plate test

Mouse hot-plate test was performed to evaluate the anti-nociceptive effects of JTC-801. Changes in the escape response latency (ERL) to heat stimulus after administration of JTC-801 are shown in the lower half of Figure 4. After intravenous administration of JTC-801, the ERL was prolonged, which indicates an anti-nociceptive effect. The prolongation was most marked at 15 min post-dosing and was statistically significant at doses of 0.01 mg kg^{-1} and above (Figure 4a). When given orally at a dosage of 1 mg kg^{-1} and above, JTC-801 prolonged the ERL with statistical significance at 90 min post-dosing (Figure 4b). In a separate experiment, the effects of morphine were examined using the same protocol. Morphine prolonged the ERL to a similar extent as JTC-801. When morphine was given intravenously (Figure 4c) or orally (Figure 4d) at a dosage of 1 mg kg^{-1} and above (i.v.) or 10 mg kg^{-1} and above (p.o.), the ERL was significantly prolonged at 15 min (i.v.) or 90 min (p.o.) post-dosing, respectively.

Effects of JTC-801 on limb licking time in rat formalin test

JTC-801 shortened the limb licking time, which indicates an anti-nociceptive effect, during both the first and second

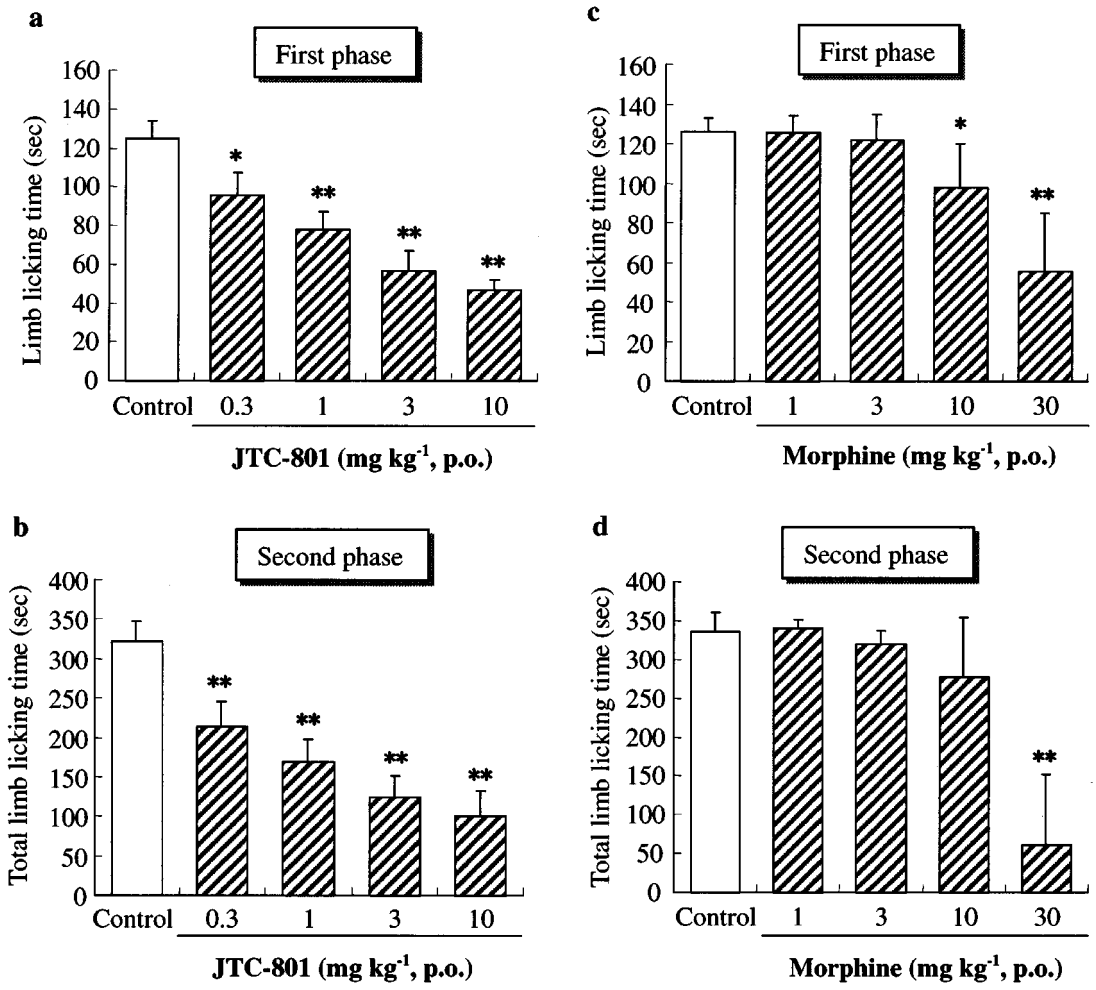

Figure 6 Effect of JTC-801 (a,b) and morphine (c,d) in the rat formalin test. JTC-801 and morphine were administered p.o. 60 min before animals received 50 μl of 5% formalin into the left hind paw. Results are expressed as mean$\pm$s.d. of time spent licking the injected paw during the first (0–5 min; a,c) and second (15–30 min; b,d) phases of seven rats. $^*P<0.05$, $^{**}P<0.01$, significantly different from vehicle treated controls (one-way ANOVA followed by Dunnett's test).

phases (Figures 5 and 6). The effect was significant at doses of 0.01 mg kg^{-1} and above when administered intravenously (Figure 5a,b). When given orally at a dosage of 0.3 mg kg^{-1} and above, JTC-801 shortened the limb licking time significantly during both the first (Figure 6a) and second (Figure 6b) phases. In a separate experiment, the effects of morphine were examined using the same protocol. Morphine also shortened the limb licking time, with significant anti-nociceptive effect at doses of 0.3 mg kg^{-1} and above during the first phase (Figure 5c) and at doses of 1.0 mg kg^{-1} and above during the second phase (Figure 5d) when administered intravenously, and at doses of 10 mg kg^{-1} and above during the first phase (Figure 6c) and at 30 mg kg^{-1} during the second phase (Figure 6d) when administered orally.

Effects of naloxone on the anti-nociceptive action of JTC-801

To investigate whether the anti-nociceptive effect of JTC-801 is mediated by opioid receptors or not, the anti-nociceptive effect of JTC-801 was examined in the presence of the opioid antagonist naloxone using rat formalin test. JTC-801 and morphine significantly shortened the duration of formalin-induced limb licking response during both of first and second phases in the absence of naloxone (Figure 7). The effect of morphine during the first and second phases was antagonized by pre-treatment with naloxone, while the anti-nociceptive effect of JTC-801 remained unaffected by pre-treatment of naloxone in both intravenous (Figure 7a,b) and oral (Figure 7c,d) administration studies.

Discussion

The present study has demonstrated that JTC-801 is a novel nociceptin receptor antagonist in *in vitro* which shows anti-nociceptin effect *in vivo*. It was shown that JTC-801 binds to ORL_1 receptor with a K_i of 44.5 nM. JTC-801 antagonized the suppression of nociceptin on forskolin-induced accumulation of cyclic AMP, indicating that JTC-801 is an antagonist of ORL_1 receptor. In the presence of JTC-801 alone, the production of cyclic AMP was not affected. These results suggest that JTC-801 does not directly affect second messengers and does not act as an agonist or partial agonist

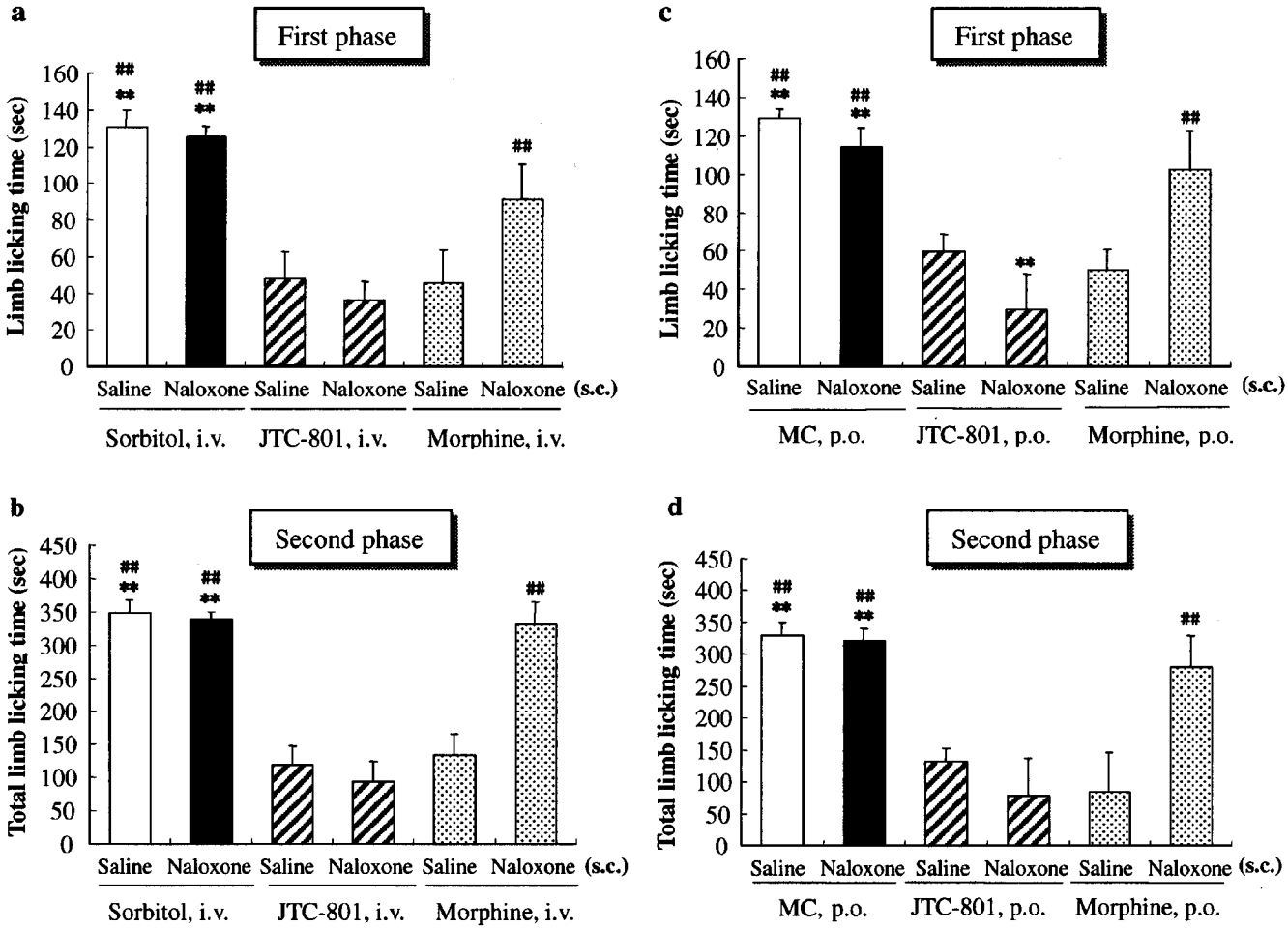

Figure 7 Effect of naloxone on the anti-nociceptive effect of JTC-801 and morphine in the formalin test. Naloxone (10 mg kg^{-1}) was administered subcutaneously 15 min before the injection of formalin. Five minutes before the injection of formalin, JTC-801 and morphine were dissolved in 5 % sorbitol and given into the tail vein at doses of 0.03 and 1.0 mg kg^{-1}, respectively (a,b). JTC-801 (3.0 mg kg^{-1}) and morphine (30 mg kg^{-1}) were administered orally 60 min before formalin injection (c,d). Results are expressed as mean ± s.d. of time spent licking the injected paw during the first (0–5 min; a,c) and second (15–30 min; b,d) phases of seven rats. $^{**}P<0.01$, significantly different from JTC-801 alone treated group, $\#\#P<0.01$, significantly different from morphine alone treated group (one-way ANOVA followed by Tukey-Kramer test).

for ORL_1 receptor. JTC-801 showed about five times higher binding affinity to human ORL_1 receptor compared to that of rat. This indicates that JTC-801 is specific to human ORL_1 receptor, and suggests a potent anti-nociceptive effect of JTC-801 in human. JTC-801 also showed specific high affinity to human ORL_1 receptor compared to other human opioid receptor subtypes. Taken together, the data indicate the receptor specificity of JTC-801. The anti-nociceptive action of JTC-801 *in vivo* is thus considered due to the antagonism of ORL_1 receptor and not to an effect on other opioid receptors such as δ-, κ-, or μ-receptor. This is also supported by the *in vivo* naloxone antagonism test, where the anti-nociceptive action of JTC-801 was not affected by naloxone.

[$Phe^1\psi(CH_2\text{-}NH)Gly^2$]NC(1–13)-$NH_2$, has been reported to be a selective antagonist of ORL_1 receptor in guinea-pig ileum and mouse vas deferens (Guerrini *et al.*, 1998). However, this pseudopeptide is also described as an agonist both *in vitro* in recombinant CHO cells expressing the ORL_1 receptor (Butour *et al.*, 1998), and *in vivo*, in the rat spinal cord (Xu *et al.*, 1998, Carpenter & Dickenson, 1998) and brain (Grisel *et al.*, 1998; Kapusta *et al.*, 1999). These different results of the functional activity of ligands to the ORL_1 receptor may depend on the type of preparation or assay used for pharmacological characterization or may be due to the pseudopeptide itself, which may be a partial agonist. The number of receptors expressed in the preparation also appears to be an important factor for the antagonistic activity of an ORL_1 receptor ligand. [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]NC(1–13)-$NH_2$, acts as an antagonist in transfected cells expressing low levels of ORL_1 receptor, while it acts as a partial or full agonist in cells expressing high receptors levels (Toll *et al.*, 1998). Contrary to [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]NC(1–13)-$NH_2$, JTC-801 dose not act as an agonist, but as an antagonist in cells expressing high receptor levels. Moreover *in vivo*, JTC-801 blocks intrathecal administered nociceptin-induced allodynia in mice. From these points of view, we have confirmed that JTC-801 is a specific human ORL_1 receptor antagonist. Nociceptin was first observed to cause thermal hyperalgesia when injected i.c.v. in mice (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). It is also reported that the effect was subsequently caused by the reversal of a stress (needleprick)-induced, opioid-mediated analgesia, rather than a decrease in nociceptive threshold (Mogil *et al.*, 1996). Thus, nociceptin may have properties of a supraspinal anti-opioid peptide. Intrathecal

injection of low (nanogram order) doses of nociceptin in mice causes allodynia, a pain response to innocuous (tactile) stimulation, while i.t. injection of even lower (picogram order) doses elicits hyperalgesia and a decrease of the nociceptive threshold, as evidenced by the hot plate test (Okuda-Ashitaka *et al*., 1996). Likewise, femtomolar i.t. doses of nociceptin elicit typical nocifensive behavior (licking, scratching and biting) in mice, which can be blocked by NK_1 receptor antagonists (Sakurada *et al*., 1999). High doses (40 μg) of i.t. nociceptin have also been reported to decrease nociceptive thresholds in rats during late gestation (Dawson-Basoa & Gintzler 1997). Thus, low doses of i.t. nociceptin may diffuse only superficially to cause allodynia and hyperalgesia, while higher dose may reach deeper into the spinal cord to produce analgesia. To further investigate of the physiological roles of nociceptin, specific antagonists are urgently needed. Towards this goal, JTC-801 represents a novel ORL_1 receptor antagonist which is the first orally available agent that is effective in *in vivo* models.

JTC-801 exerted an anti-nociceptive effect in a noxious thermal pain model (hot plate test) and in an inflammatory pain model (formalin test) by both intravenous and oral administration. In comparison with morphine in these models, the potency of JTC-801 was higher and the efficacy was more than equivalent. Furthermore, the anti-nociceptive effect of JTC-801 was not antagonized by naloxone (10 mg kg^{-1}), an opioid antagonist, in the formalin test. This result suggests that, unlike morphine, JTC-801 exerts its anti-nociceptive effect not *via* opioid receptors. Interestingly, another low affinity ORL_1 receptor antagonist, retronociceptin methyl-ester, was reported to act as an analgesic following i.c.v. administration in mice (Yoshikawa *et al*., 1999), and a similar result was also obtained using naloxone benzoylhydrazone in wild type but not in nociceptin receptor knockout mice (Noda *et al*., 1998). Recently, another pseudopeptide, $[Nphe^1]NC(1-13)NH_2$, was reported to be a selective ORL_1 receptor antagonist both at recombinant and native ORL_1 sites. This new pseudopeptide prevented the pronociceptive and antimorphine actions of i.c.v. applied nociceptin, and produced a naloxone resistant anti-nociceptive action (Calo' *et al*., 2000). This evidence suggests that endogenous nociceptin plays a role in the modulation of pain and the block of nociceptin signaling raises pain threshold. From these points of view, JTC-801, a specific nociceptin receptor antagonist, may represent a new class of anti-nociceptive drug, which inhibits nociception like morphine, but does not have the adverse effects of morphine such as drug dependence and tolerance.

In summary, we have demonstrated that JTC-801 is the first orally active ORL_1 receptor antagonist, and that JTC-801 has efficacious and potent anti-nociceptive effects in acute pain models. We also confirmed that the anti-nociceptive effect of JTC-801 is not *via* opioid receptors, and thus JTC-801 may not have the side effects typically associated the opioid analgesics. Taken together, these findings suggest that JTC-801 may represent a new class of analgesics.

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