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Pharmacological properties of TRK-820 on cloned μ-, δ- and κ-opioid receptors and nociceptin receptor

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Abstract

We analyzed the pharmacological properties of 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[*N*-methyl-*trans*-3-(3-furyl)acrylamido]morphinan hydrochloride (TRK-820) using Chinese hamster ovary (CHO) cells expressing cloned rat μ-, δ- and κ-opioid receptors and human nociceptin receptor. TRK-820 showed high affinity for the κ-opioid receptor, with a K_i value of 3.5 ± 0.9 nM. In CHO cells expressing κ-opioid receptors, TRK-820 inhibited forskolin-stimulated cAMP accumulation, and the maximal inhibitory effect was equivalent to that of (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro-(4,5)dec-8-yl]benzeneacetamide (U69,593), a full agonist of κ-opioid receptor. In CHO cells expressing μ-opioid receptors, TRK-820 inhibited cAMP accumulation, but the maximal inhibitory effect was significantly smaller than that of [D-Ala^2, *N*-MePhe^4, Gly-ol^5]enkephalin (DAMGO), a full agonist of μ-opioid receptor. In CHO cells expressing δ-opioid receptor, the inhibitory effect of TRK-820 on cAMP accumulation was very weak. Using site-directed mutagenesis, the high affinity of TRK-820 for the κ-opioid receptor was revealed to require Glu^{297}. TRK-820 bound to the nociceptin receptor with a K_i value of 380 ± 50 nM. TRK-820 by itself had no effect on cAMP accumulation in CHO cells expressing nociceptin receptors, but significantly antagonized the nociceptin (10 nM)-mediated inhibition of cAMP accumulation at high concentrations. These results indicate that TRK-820 acts as a full agonist for the κ-opioid receptor, a partial agonist for the μ-opioid receptor and a low-affinity antagonist for the nociceptin receptor.

Keywords: TRK-820; Opioid receptor; Nociceptin receptor; Binding selectivity; Agonistic activity; Antagonistic activity

1. Introduction

The opioid receptors are classified into at least three types, that is μ-, δ- and κ-types, and the pharmacological properties of each type have been studied by using several type-selective agonists and antagonists (Satoh and Minami, 1995; Dhawan et al., 1996). As to κ-opioid receptor, benzeneacetamide compounds, represented by U50,488H ((±)-*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide) and U69,593 ((+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetamide) were synthesized and have been used as κ-opioid receptor-selective agonists (Piercey et al., 1982; Lahti et al., 1985; Costello et al., 1988). For a κ-opioid receptor-selective antagonist, norbinaltorphimine (Fig. 1) was synthesized according to the ''message-address'' concept (Portoghese et al., 1987). Recently, TRK-820 (17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[*N*-methyl-*trans*-3-(3-furyl)acrylamido]morphinan hydrochloride (Fig. 1) has been synthesized as a novel κ-opioid receptor-selective agonist by removing the ''accessory site'' from norbinaltorphimine (Nagase et al., 1998). TRK-820 was reported to have a potent and κ-opioid receptor-selective agonistic activity in both in vitro studies with guinea pig ileum and mouse vas deferens and in vivo studies including acetic acid writhing test and tail flick test in mice. Furthermore, TRK-820 neither induced aversive nor reinforcing effects in a conditioned place preference experiment (Nagase et al., 1998). These findings suggest that TRK-820 could be an ideal analgesic which has no psychological effects. To investigate further the pharmacological properties of TRK-820, in this study, we examined the binding profile and agonistic activity of TRK-820 in

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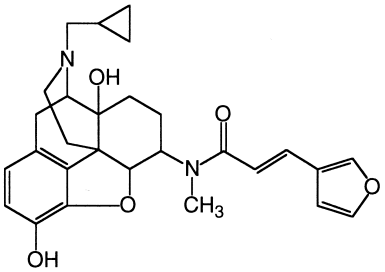
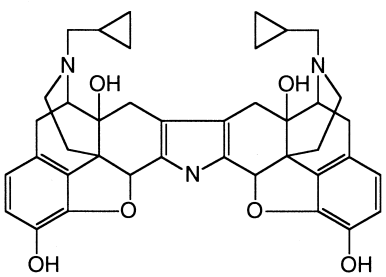


TRK-820



norbinaltorphimine

Fig. 1. Structures of TRK-820 and norbinaltorphimine.

Chinese hamster ovary (CHO) cells expressing the cloned μ-, δ- or κ-opioid receptors.

It was revealed that norbinaltorphimine distinguishes the κ-opioid receptor from the μ-opioid receptor via the difference between corresponding amino acids, Glu^{297} of the κ-opioid receptor and Lys^{303} of the μ-opioid receptor located in the boundary region between the third extracellular loop and the sixth transmembrane domain (Hjorth et al., 1995). Since TRK-820 is a derivative of norbinaltorphimine, we supposed that the selectivity of TRK-820 for the κ-opioid receptor might be also influenced by the same amino acid residue. We therefore constructed several mutant opioid receptors to examine whether the same residue is important for the binding selectivity of TRK-820 for the κ-opioid receptor.

A novel receptor ORL1 (opioid receptor-like 1), belonging to the opioid receptor family due to its close similarity in amino acid sequence with μ-, δ- and κ-opioid receptors, was cloned (Fukuda et al., 1994; Mollereau et al., 1994), but had been regarded as an orphan receptor because no known opioid peptides or alkaloids bound to this receptor with high affinity. Recently, an endogenous peptidic agonist of ORL1 was identified by two independent groups and termed nociceptin/orphanin FQ (Meunier et al., 1995; Reinscheid et al., 1995). It has been reported that i.c.v. administration of nociceptin induced hyperalgesia or anti-opioid effect (Meunier et al., 1995; Reinscheid et al., 1995; Mogil et al., 1996). On the other hand, i.t. injection of this peptide produced antinociceptive and hyperalgesic effects depending on the doses (Okuda-Ashitaka et al., 1996; Xu et al., 1996). These findings suggest that nociceptin is involved in pain transmission and/or regulation systems, but its exact role remains unclear. To fully elucidate the physiological and pathological roles of nociceptin, nonpeptidic ligands for the nociceptin receptor are considered to be useful, but such ligands have not been obtained yet. Recently, Noda et al. (1998) have reported that naloxone benzoylhydrazone, which has been called an agonist for the κ_3-opioid receptor, also antagonized the nociceptin-mediated inhibition of cAMP accumulation in CHO expressing the nociceptin receptor at high dose. If TRK-820 has a more potent agonistic or antagonistic activity for the nociceptin receptor, it may be a leading compound of the nociceptin receptor-selective nonpeptidic ligand. Therefore, we constructed CHO cells stably expressing nociceptin receptors and examined the binding profile and agonistic/antagonistic activity of TRK-820 to the nociceptin receptor and compared them with those of naloxone benzoylhydrazone.

2. Materials and methods

2.1. Materials

The rat μ- and κ-opioid receptor cDNAs were cloned as previously described (Minami et al., 1993, 1994). The rat δ-opioid receptor cDNA (Fukuda et al., 1993) was a gift from Dr. K. Fukuda. The human nociceptin receptor cDNA (Seki et al., 1998a) was cloned from HL60 cells, a human myeloid cell line, by a reverse transcription-polymerase chain reaction (RT-PCR) based method. [D-Ala^2, $MePhe^4$, $Gly\text{-}ol^5$]enkephalin (DAMGO), a μ-opioid receptor-selective agonist, and [D-Pen^2, D-Pen^5]enkephalin (DPDPE), a δ-opioid receptor-selective agonist, were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). A κ-opioid receptor-selective agonist, U69,593 was a gift from the Upjohn (Kalamazoo, MI, USA). Nociceptin was purchased from Peninsula Laboratories, (Belmont, CA, USA). TRK-820, norbinaltorphimine and naloxone benzoylhydrazone were synthesized by Dr. H. Nagase. [^{3}H]Bremazocine (26.6 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA, USA), and [^{3}H]nociceptin (163 Ci/mmol) from Amersham (Buckinghamshire, UK).

2.2. Construction of mutant opioid receptors and expression of wild type and mutant opioid receptors and nociceptin receptor on CHO cells

In vitro site-directed mutagenesis was carried out using a Transformer™ Site-Directed Mutagenesis Kit (2nd version) (CLONTECH Laboratories, Palo Alto, CA, USA) as described (Seki et al., 1998b). The fragment containing the full length coding region of each wild type or mutant receptor cDNA was subcloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA). CHO cells

were grown in F-12 medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C. The cells were transfected by the lipofectin method with the plasmids, and selected by cultivation in the presence of 500 μg/ml of G418 (GIBCO BRL, Gaithersburg, NY, USA). Cells expressing each opioid receptor or nociceptin receptor were selected by a binding assay with [^{3}H]bremazocine, a nonselective opioid ligand, or by the inhibition of cAMP accumulation in the presence of 10 nM nociceptin, respectively. CHO cells stably expressing wild type μ-, δ-, κ-opioid receptor, nociceptin receptor or mutant receptor were named as CHO/MOPR, CHO/DOPR, CHO/KOPR, CHO/NociR or CHO/(mutant receptor), respectively.

2.3. Radioligand binding assay

Binding was assayed as described previously (Katsumata et al., 1995). In brief, cells were harvested and homogenized in binding buffer containing 50 mM Tris (pH 7.4), 10 mM $MgCl_2$ and 1 mM EDTA. For the nociceptin receptor, the buffer was supplemented by 0.1% bovine serum albumin. After centrifugation for 20 min at 30,000 $\times g$, the pellets were resuspended in binding buffer and used as membrane preparations. For saturation binding assay, the cell membrane preparations were incubated for 60 min at 25°C with various concentrations of [^{3}H]bremazocine for opioid receptors or [^{3}H]nociceptin for nociceptin receptor. Nonspecific binding was determined in the presence of 10 μM unlabeled bremazocine or nociceptin, respectively. For competitive binding assay, the cell membrane preparations were incubated for 60 min at 25°C with 1–4 nM [^{3}H]bremazocine or 0.5 nM [^{3}H]nociceptin in the presence of various concentrations of ligands. All of ligands except for naloxone benzoylhydrazone were dissolved in the binding buffer. Since naloxone benzoylhydrazone hardly dissolved in aqueous solution, it was dissolved and diluted in 0.05 N acetic acid containing 20% dimethyl sulfoxide, and then added to the incubation mixture. Final concentration of vehicle was 0.005 N acetic acid/2% dimethyl sulfoxide in binding buffer. These concentrations of actic acid and dimethyl sulfoxide did not affect the binding of [^{3}H]nociceptin. After incubation for 60 min, the membrane preparations were rapidly filtrated over Whatman GF/C glass fiber filters which were pretreated with 0.1% or 0.3% polyethyleneimine for opioid receptors or nociceptin receptor, respectively, and the radioactivity on each filter was measured by liquid scintillation counting. K_d values of the radiolabeled ligands were obtained by Scatchard analyses. K_i values were calculated from the IC_{50} values obtained from the competitive binding assay in accordance with the equation $K_i = IC_{50}/(1 + [\text{radiolabeled ligand}]/K_d)$ (Cheng and Prusoff, 1973), where IC_{50} is the concentration of unlabeled ligand required to displace 50% inhibition of the radiolabeled ligand. The results of binding assays are presented as the mean ± S.E. of 3–5 separate experiments.

2.4. cAMP assay

cAMP assay was performed as described previously (Katsumata et al., 1995). Cells were seeded into 24-well plates at a density of 10^5 cells/well. After cultivation for 24 h, the cells were washed with 0.5 ml of HEPES-buffered saline (140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 15 mM HEPES, pH 7.4) and incubated with 0.45 ml of HEPES-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37°C. 3-Isobutyl-1-methylxanthine was dissolved in dimethyl sulfoxide and then added to the incubation mixture, where the final concentration of dimethyl sulfoxide was 0.2%. Then, the stimulation was started by the addition of 50 μl of HEPES-buffered saline containing 100 μM forskolin (final concentration = 10 μM) and 1 mM 3-isobutyl-1-methylxanthine in the presence or absence of various concentrations of opioids. All of the drugs except for naloxone benzoylhydrazone were dissolved in HEPES-buffered saline containing 100 μM forskolin and 1 mM 3-isobutyl-1-methylxanthine. Since naloxone benzoylhydrazone hardly dissolved in aqueous solution, it was dissolved and diluted in 0.05 N acetic acid containing 20% dimethyl sulfoxide, and then added to the incubation mixture. Final concentration of vehicle was 0.0025 N acetic acid/1% dimethyl sulfoxide. After incubation for 10 min, the stimulation was terminated by the addition of an equal amount of ice-cold 10% trichloroacetic acid to each well. The concentration of cAMP was measured using a radioimmunoassay kit (Amersham, Buckinghamshire, UK). The amount of cAMP in the control (without any opioids) was 76 ± 5 pmol/well, and the data of cAMP accumulation are presented as percent of control. In the case of experiments with naloxone benzoylhydrazone, additional vehicle 0.0025 N acetic acid/1% dimethyl sulfoxide decreased the production of cAMP to 48 ± 11 pmol/well, and this value was used as a control to obtain the data of cAMP accumulation (percent of control) throughout the experiments with naloxone benzoylhydrazone. IC_{50} values were calculated as the concentrations of ligands producing 50% of the maximal inhibition in cAMP accumulation. The values of IC_{50} and maximal inhibitory effect (I_{max}) in cAMP assays are presented as the mean ± S.E. of 3–5 separate experiments performed in triplicate.

3. Results

3.1. Profile of TRK-820 binding to the membrane preparations from CHO / MOPR, CHO / DOPR and CHO / KOPR

CHO cell lines that stably express cloned μ-, δ- and κ-opioid receptors (CHO/MOPR, CHO/DOPR and CHO/KOPR, respectively) were established. In the saturation binding assay, [^{3}H]bremazocine, a radiolabeled nonse-

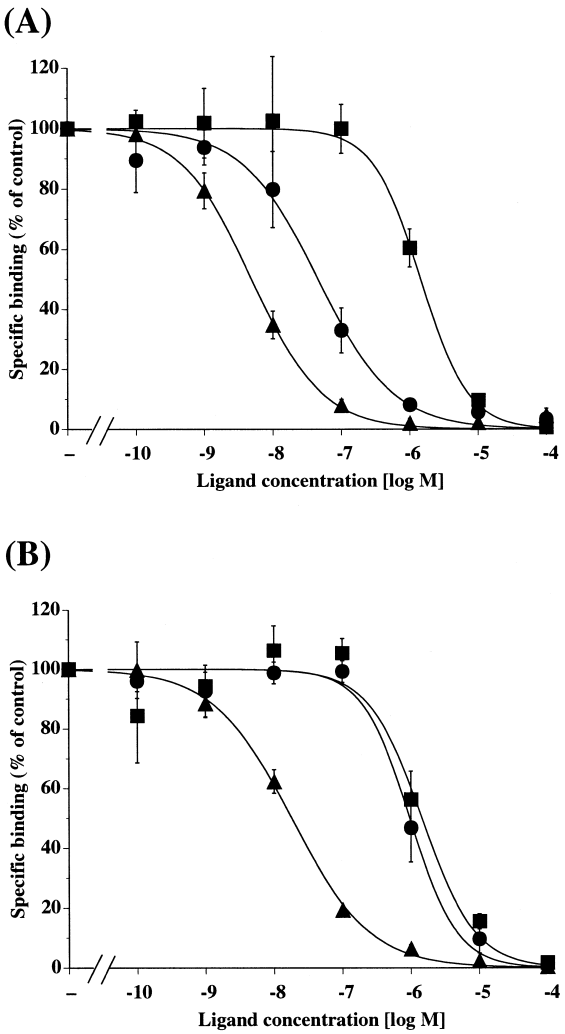


Fig. 2. Competitive inhibition of specific binding of [^{3}H]bremazocine by TRK-820 (A) and norbinaltorphimine (B) in the membrane preparations from CHO/MOPR (●), CHO/DOPR (■) and CHO/KOPR (▲). The data are presented as means ± S.E.M. of 3–5 separate experiments.

lective opioid ligand, bound with high affinity to the membrane preparations from these cells and the calculated K_d values of [^{3}H]bremazocine to the μ-, δ- and κ-opioid receptors were 2.5 ± 0.6, 4.8 ± 0.8 and 1.8 ± 0.6 nM, respectively. The receptor densities (B_{max}) in these cells were 1400 ± 220, 7400 ± 890 and 2100 ± 350 fmol/mg protein, respectively.

In the competition binding assay, TRK-820 bound to the κ-opioid receptor with high affinity and the calculated K_i value was 3.5 ± 0.9 nM, which was slightly smaller than that of norbinaltorphimine ($K_i = 8.7 \pm 1.2$ nM). The affinity of TRK-820 for the μ-opioid receptor was lower than that for the κ-opioid receptor and the calculated K_i value was 53 ± 12 nM, which was about 15-fold smaller than that of norbinaltorphimine ($K_i = 800 \pm 240$ nM). The binding selectivity of TRK-820 to the κ-opioid receptor over the μ-opioid receptor (about 15-fold) was lower than that of norbinaltorphimine (about 90-fold). Both TRK-820 and norbinaltorphimine bound to the δ-opioid receptor with low affinities, and the calculated K_i values were 1200 ± 300 and 1500 ± 500 nM, respectively. Their selectivities to the κ-opioid receptor over the δ-opioid receptor (about 330 fold and 170 fold, respectively) were higher than those to the κ-opioid receptor over the μ-opioid receptor (Fig. 2 and Table 1).

3.2. Agonistic activity of TRK-820 in CHO / MOPR, CHO / DOPR and CHO / KOPR

Agonistic activity of TRK-820 was evaluated by the inhibitory effect it had on forskolin (10 μM)-stimulated intracellular cAMP accumulation. TRK-820 inhibited forskolin-stimulated intracellular cAMP accumulation in a concentration-dependent manner in CHO/KOPR (Fig. 3A). The IC_{50} value of TRK-820 was 0.15 ± 0.08 nM, which was about 100 fold smaller than that of U69593, a κ-opioid receptor-selective agonist ($IC_{50} = 16 \pm 6$ nM). The I_{max} values of TRK-820 and U69593 in CHO/KOPR were $81 \pm 3\%$ and $72 \pm 5\%$, respectively, and there was no significant difference between the maximal inhibitory effects of TRK-820 and U69,593. In CHO/MOPR, TRK-820 inhibited forskolin-stimulated cAMP accumulation with an IC_{50} value of 8.3 ± 1.4 nM, similar to that of DAMGO, a μ-opioid receptor selective agonist ($IC_{50} = 5.0 \pm 1.1$ nM; Fig. 3B). However, the I_{max} value of TRK-820 in CHO/MOPR ($69 \pm 3\%$) was significantly smaller than that of DAMGO ($88 \pm 1\%$, $P < 0.01$ in Student's *t*-test). In CHO/DOPR, DPDPE inhibited forskolin-stimulated cAMP accumulation with an IC_{50} value of 5.4 ± 1.7 nM and I_{max} value of $78 \pm 3\%$. On the other hand, the inhibitory effect of TRK-820 on cAMP accumulation in CHO/DOPR was very weak ($IC_{50} > 1000$ nM) and did not reach maximum at the highest concentration (10 μM) used in this study (Fig. 3C).

3.3. Profile of TRK-820 and norbinaltorphimine binding to the membrane preparations from CHO cells expressing mutant opioid receptors

We constructed κ(E297K), in which Glu^{297} of the κ-opioid receptor was mutated into Lys found at the

Table 1
K_i values (nM) of TRK-820 and norbinaltorphimine in the membrane preparations of CHO cells expressing wild type or mutant opioid receptors

	TRK-820	Norbinaltorphimine
κ	3.5 ± 0.9	8.7 ± 1.2
μ	53 ± 12	800 ± 240
δ	1200 ± 300	1500 ± 500
κ(E297K)	18 ± 4	1900 ± 400
μ(K303E)	5.5 ± 0.9	29 ± 5
κ(E297W)	53 ± 13	1000 ± 300
δ(W284E)	> 3000	1000 ± 300
κ(E297A)	12 ± 3	300 ± 60

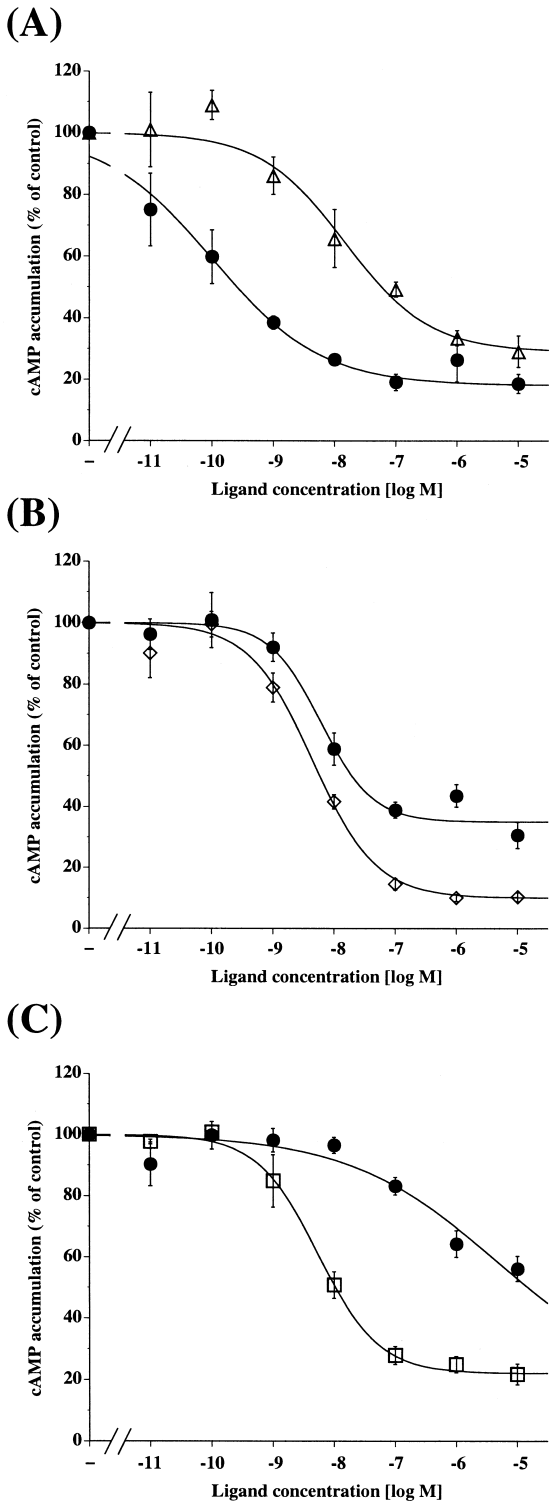


Fig. 3. Effects of opioids on forskolin (10 μM)-induced cAMP accumulation in CHO/KOPR (A), CHO/MOPR (B) and CHO/DOPR (C). Opioid ligands used were TRK-820 (●), U69,593 (△), DAMGO (◇) and DPDPE (□). Results are presented as means ± S.E.M. of 3–5 separate experiments performed in triplicate.

corresponding position of the μ-opioid receptor, and μ(K303E), in which Lys^{303} of the μ-opioid receptor was mutated into Glu. [^{3}H]Bremazocine bound to both κ(E297K) and μ(K303E) with high affinities ($K_d = 1.9 \pm 1.2$ and 5.4 ± 4.0 nM, respectively). The B_{max} values in these cells were 2700 ± 1300 and 2100 ± 1500 fmol/mg protein, respectively. The affinities of TRK-820 and norbinaltorphimine for κ(E297K) ($K_i = 18 \pm 4$ and 1900 ± 400 nM, respectively) were decreased from those for the wild-type κ-opioid receptor. Conversely, the affinities of TRK-820 and norbinaltorphimine for μ(K303E) ($K_i = 5.5 \pm 0.9$ and 29 ± 5 nM, respectively) were increased from those for the wild-type μ-opioid receptor and were comparable to those for the κ-opioid receptor.

To examine whether Glu^{297} of the κ-opioid receptor is the determinant for the discrimination between κ- and δ-opioid receptors by TRK-820 and norbinaltorphimine, we constructed κ(E297W), in which Glu^{297} of the κ-opioid receptor was mutated into Trp found at the corresponding position of the δ-opioid receptor, and δ(W284E), in which Trp^{284} of the δ-opioid receptor was mutated into Glu. [^{3}H]Bremazocine bound to these mutant receptors with high affinities ($K_d = 7.3 \pm 1.6$ and 6.5 ± 1.5 nM, respectively) and the B_{max} values were 860 ± 170 and 1100 ± 300 fmol/mg protein, respectively. The affinities of TRK-820 and norbinaltorphimine for κ(E297W) ($K_i = 53 \pm 13$ and 1000 ± 300 nM, respectively) were decreased from those for the κ-opioid receptor. However, different from the case of μ(K303E), the affinities for TRK-820 and norbinaltorphimine for δ(W284E) were very low ($K_i >$ 3000 and 1000 ± 300 nM, respectively) and comparable to those for the δ-opioid receptor.

Furthermore, to investigate the role of Glu^{297} of the κ-opioid receptor in the selectivity for the κ-opioid receptor of TRK-820 and norbinaltorphimine, we constructed κ(E297A), in which Glu^{297} of the κ-opioid receptor was mutated into Ala, which has a small and neutral side chain. [^{3}H]Bremazocine bound to κ(E297A) with a high affinity ($K_d = 1.1 \pm 0.5$ nM) and the B_{max} value was 840 ± 46 fmol/mg protein. The affinities of TRK-820 and norBNI for κ(E297A) ($K_i = 12 \pm 3$ and 300 ± 60 nM, respectively) were lower than those for the κ-opioid receptor (Table 1).

3.4. Binding profile and agonistic / antagonistic activity of TRK-820 and naloxone benzoylhydrazone in CHO / NociR

We constructed CHO cells expressing nociceptin receptors (CHO/NociR). In the saturation binding assay, [^{3}H]nociceptin bound to the nociceptin receptor with a high affinity ($K_d = 0.19 \pm 0.04$ nM) and the B_{max} value was 61 ± 8 fmol/mg protein. In the competitive binding assay, TRK-820 and naloxone benzoylhydrazone bound to the nociceptin receptor with relatively low affinities, and the calculated K_i values were 380 ± 50 and 4.2 ± 1.0 nM, which were about 1000 and 10 times lower than that of nociceptin ($K_i = 0.35 \pm 0.09$ nM, Fig. 4A), respectively.

To evaluate the agonistic activities of TRK-820 and naloxone benzoylhydrazone in CHO/NociR, the effects of

various concentrations of them on forskolin-stimulated intracellular cAMP accumulation were examined. As shown in Fig. 4B, TRK-820 and naloxone benzoylhydrazone (from 100 pM to 100 μM) did not show any apparent inhibitory effects on forskolin-stimulated cAMP accumulation in CHO/NociR, although nociceptin inhibited forskolin-stimulated cAMP accumulation in a concentration-dependent manner ($IC_{50} = 0.18 \pm 0.04$ nM and $I_{max} = 76 \pm 3\%$). Furthermore, the antagonistic activity of TRK-820 in CHO/NociR was evaluated by the effects of various concentrations of TRK-820 on the nociceptin (10 nM)-mediated inhibition of cAMP accumulation. Nociceptin (10 nM) inhibited the cAMP accumulation to $27 \pm 1\%$ of the control level. TRK-820 significantly antagonized the nociceptin (10 nM)-mediated inhibition of cAMP accumulation at concentrations of 10 and 100 μM ($47 \pm 4\%$ and $67 \pm 8\%$ of the control level, respectively; Fig. 4C). Naloxone benzoylhydrazone significantly antagonized the nociceptin (10 nM)-mediated inhibition of cAMP accumulation at a concentration of 100 μM ($88 \pm 6\%$ of the control level, Fig. 4D).

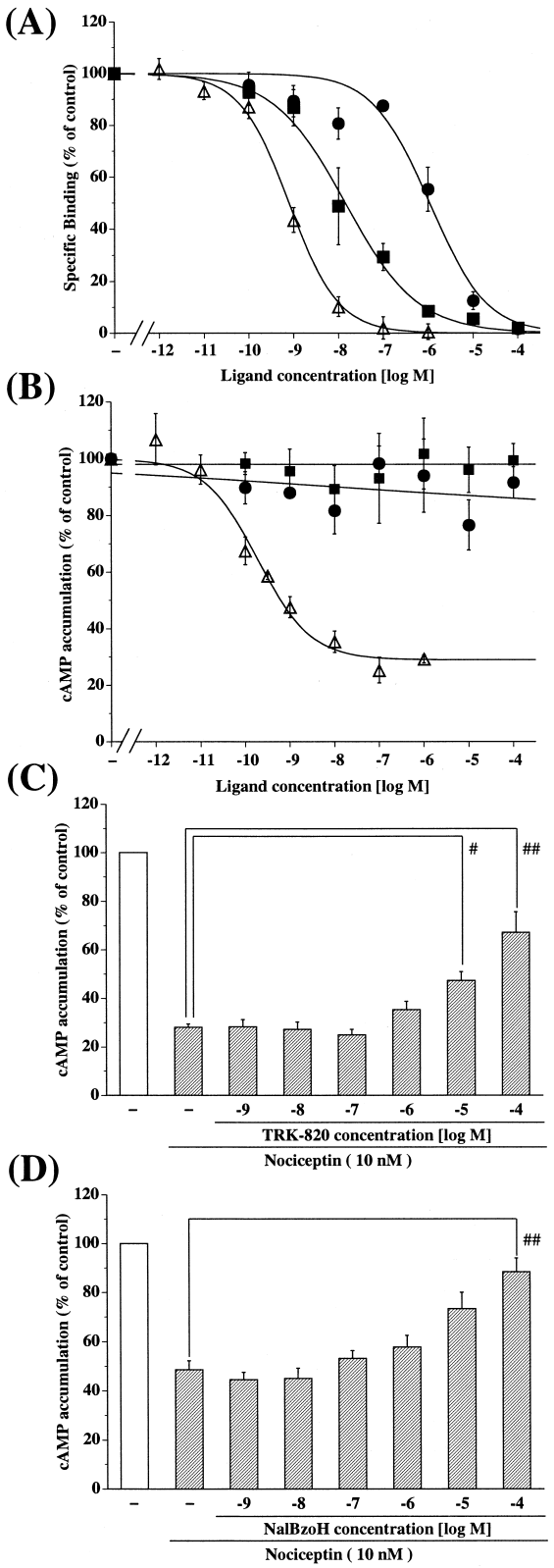


Fig. 4. (A) Competitive inhibition of specific binding of [^{3}H]nociceptin by TRK-820 (●), naloxone benzoylhydrazone (■) and nociceptin (△) in the membrane preparations from CHO/NociR. (B) Effects of TRK-820 (●), naloxone benzoylhydrazone (■) and nociceptin (△) on forskolin (10 μM)-induced cAMP accumulation in CHO/NociR. (C,D) Effects of TRK-820 (C) and naloxone benzoylhydrazone (D) on nociceptin (10 nM)-mediated inhibition of cAMP accumulation in CHO/NociR. Results are presented as means ± S.E.M. of 3–5 separate experiments. $\#P < 0.05$; $\#\#P < 0.01$ compared with nociceptin (10 nM) alone (Dunnett's test).

4. Discussion

TRK-820 has been synthesized as a new κ-opioid receptor-selective agonist, and reportedly it has a more potent antinociceptive effect than morphine and U69,593 in mice, and potent agonistic activities in guinea pig ileum and mouse vas deferens (Nagase et al., 1998). The antinociceptive effects were antagonized by norbinaltorphimine, a κ-opioid receptor antagonist, but not by naloxone nor naltrindol which preferentially bind μ- or δ-opioid receptor, respectively (Katsumata et al., 1995). The other agonistic activities of TRK-820 were antagonized by norbinaltorphimine and the antagonistic potency of norbinaltorphimine was more than 100 times that of naloxone and naltrindol in guinea pig ileum and mouse vas deferens. In this study, we firstly examined the affinities of TRK-820 for the μ-, δ- and κ-opioid receptors by competition binding assay in the membrane preparations from CHO cells expressing one of the cloned opioid receptors. We showed that the K_i values of TRK-820 for the κ-, μ- and δ-opioid receptors were 3.5 ± 0.9, 53 ± 12 and 1200 ± 300 nM, respectively. TRK-820 had about 15 and 340 times higher affinity for the κ-opioid receptors than for the μ- and δ-opioid receptors, respectively. The binding selectivity of TRK-820 for the κ-opioid receptor over the μ-opioid receptor was much smaller than the selectivity of

the agonistic activity from studies in guinea pig ileum and mouse vas deferens (Nagase et al., 1998). In this study, we also showed that the K_i values of norbinaltorphimine for the κ-, μ- and δ-opioid receptors were 8.7 ± 1.2, 800 ± 240 and 1500 ± 500 nM, respectively. Furthermore, we previously reported that the K_i values of U69.593 for the κ-, μ- and δ-opioid receptors were 12 ± 7, > 3000 and > 3000 nM, respectively (Katsumata et al., 1995). The binding selectivity of TRK-820 for the κ-opioid receptor over the μ-opioid receptor was lower than those of norbinaltorphimine and U69,593 (about 90 and > 250 times higher affinity for the κ-opioid receptor than for the μ-opioid receptor, respectively).

In the present study, we examined agonistic activities of TRK-820 in CHO/KOPR, CHO/MOPR and CHO/DOPR. In CHO/KOPR, TRK-820 and U69,593 inhibited forskolin-stimulated cAMP accumulation with IC_{50} values of 0.15 ± 0.07 and 16 ± 6 nM, respectively. TRK-820 was about 100 times more potent in inhibiting the cAMP accumulation than U69,593. The I_{max} value of TRK-820 was equivalent to that of U69,593 (81 ± 3 and 72 ± 5%, respectively), suggesting that TRK-820 has a full agonistic activity for the κ-opioid receptor. These results indicate that TRK-820 is a very potent agonist for the κ-opioid receptor. In CHO/MOPR, TRK-820 and DAMGO inhibited forskolin-stimulated cAMP accumulation with IC_{50} values of 8.3 ± 1.4 and 5.0 ± 1.5 nM, respectively. However, the I_{max} value of TRK-820 (69 ± 3%) was significantly smaller than that of DAMGO (88 ± 1%), suggesting TRK-820 acts as a partial agonist for the μ-opioid receptor. The differences in IC_{50} values (about 55 times) and maximal inhibitory effects of TRK-820 between CHO/KOPR and CHO/MOPR may contribute to the agonistic activity of TRK-820 selective for the κ-opioid receptor over the μ-opioid receptor in guinea pig ileum and mouse vas deferens (Nagase et al., 1998). In CHO/DOPR, DPDPE inhibited forskolin-stimulated cAMP accumulation with an IC_{50} value of 5.4 ± 1.7 nM and I_{max} value of 78 ± 3%. Although TRK-820 also inhibited cAMP accumulation in a concentration-dependent manner, its inhibitory effect did not reach maximum even at a concentration of 10 μM, the highest concentration used in this study, suggesting that TRK-820 is a very weak agonist for the δ-opioid receptor. Taken together, these results revealed that TRK-820 may act as a very potent full agonist for the κ-opioid receptor, a partial agonist for the μ-opioid receptor and a very weak agonist for the δ-opioid receptor in the CHO cells expressing each type of the opioid receptor. Such an in vitro profile of TRK-820 is similar to that of mixed agonist/antagonists for opioid receptors including pentazocine, butorphanol and KT-90 ((−)-3-acetyl-6β-acetylthio-*N*-cyclopropylmethyl-normorphine) (Cherny, 1996; Katsumata et al., 1996). Furthermore, DeHaven et al. (1998) recently reported that TRK-820 showed an agonistic activity for the κ-opioid receptor and an antagonistic activity for the μ-opioid receptor in a GTPγS binding assay using CHO cells expressing each type of opioid receptor. On the other hand, there is as yet no evidence that the μ-opioid receptor-mediated activity contributes to any of the in vivo pharmacological actions of TRK-820 (e.g., TRK-820 slightly augmented morphine analgesia, whereas pentazocine reduced it; Endoh et al., personal communications), suggesting TRK-820 has different characteristics to the preexisting mixed agonist/antagonists for opioid receptors.

Hjorth et al. (1995) reported that the affinity of norbinaltorphimine for the κ-opioid receptor was markedly decreased by the replacement of Glu^{297} located in the boundary region between the sixth transmembrane domain and the third extracellular loop of the κ-opioid receptor with Lys located at the corresponding position of the μ-opioid receptor. In the present study, we constructed several mutant receptors and examined whether the same residue was critical for the selectivity of TRK-820 binding. The affinities of TRK-820 and norbinaltorphimine for κ(E297K), in which Glu^{297} of the κ-opioid receptor was replaced with Lys, were about 5 and 218 times less, respectively, than those for the wild type κ-opioid receptor. In the mutant receptor μ(K303E), in which Lys^{303} of the μ-opioid receptor was mutated into Glu, the affinities of TRK-820 and norbinaltorphimine increased about 10- and 27-fold, respectively, from those for the wild type μ-opioid receptor. These results indicate that the difference between Glu^{297} of the κ-opioid receptor and Lys^{303} of the μ-opioid receptor is important for the discrimination between κ- and μ-opioid receptors by TRK-820 and norbinaltorphimine, but that difference has much more influence on the discrimination by norbinaltorphimine than by TRK-820. To further evaluate the necessity of Glu^{297} for that discrimination by TRK-820 and norbinaltorphimine, we mutated this residue of the κ-opioid receptor into Ala, which has a small and neutral side chain. For the resultant mutant receptor, κ(E297A), the affinities of TRK-820 and norbinaltorphimine were about 3 and 35 times, respectively, lower than those for the wild type κ-opioid receptor. This result supported that Glu^{297} is more necessary for the high affinity binding of norbinaltorphimine to the κ-opioid receptor than that of TRK-820. It has been reported that basic N17′ nitrogen of norbinaltorphimine contributes to κ-opioid receptor-selective and potent antagonistic activity (Portoghese et al., 1994). Since Glu is an acidic amino acid residue, the basic nitrogen of norbinaltorphimine at N17′ may directly interact with Glu^{297} of the κ-opioid receptor. TRK-820 lacks for the nitrogen corresponding to N17′ of norbinaltorphimine (Fig. 1). This may be the reason for the much more importance of Glu^{297} of the κ-opioid receptor in κ-selective binding of norbinaltorphimine than that of TRK-820. To investigate whether the residue at the corresponding position of δ-opioid receptor is involved in the discrimination between κ- and δ-opioid receptors by TRK-820 and norbinaltorphimine, we constructed two other mutant receptors,

κ(E297W) and δ(W284E). The affinities of TRK-820 and norbinaltorphimine for κ(E297W) were about 15 and 120 times, respectively, lower than those for the wild type κ-opioid receptor. However, for δ(W284E), the affinities of TRK-820 and norbinaltorphimine did not increase and were still low compared to those for the wild type δ-opioid receptor. These results suggest that the difference between Glu^{297} of the κ-opioid receptor and Trp^{284} of the δ-opioid receptors is not so critical for the discrimination between κ- and δ-opioid receptors by these compounds, and there is another region(s) involved in this discrimination.

The affinity of TRK-820 for the nociceptin receptor ($K_i = 380 \pm 50$ nM) was about 1000 times lower than that of nociceptin ($K_i = 0.35 \pm 0.09$ nM). This affinity of TRK-820, however, was relatively high among most of the opioid ligands, equivalent to that of etorphine and lower than that of naloxone benzoylhydrazone shown in our study and those of dynorphin A and lofentanil reported by Meng et al. (1996) and Mollereau et al. (1996). To evaluate whether TRK-820 acts on the nociceptin receptor as an agonist or an antagonist, the effect of TRK-820 on forskolin-stimulated intracellular cAMP accumulation in CHO/NociR was examined and compared with those of nociceptin and naloxone benzoylhydrazone, the latter of which was reported to have an antagonistic activity on the nociceptin receptor at relatively high dose (Noda et al., 1998). TRK-820 applied alone did not have any significant effects on cAMP accumulation in CHO/NociR at concentrations from 100 pM to 100 μM, suggesting that TRK-820 has no agonistic activity for the nociceptin receptor. On the other hand, 10 and 100 μM TRK-820 significantly antagonized the nociceptin (10 nM)-mediated inhibition of cAMP accumulation. Similarly, 100 μM naloxone benzoylhydrazone significantly antagonized the nociceptin (10 nM)-mediated inhibition of cAMP accumulation in CHO/NociR, without any significant effects on cAMP accumulation by itself. These results indicate that TRK-820 acts on the nociceptin receptor as a low-affinity antagonist and the antagonistic potency of TRK-820 is equivalent to that of naloxone benzoylhydrazone. Noda et al. (1998) have also reported that the antinociceptive effects of naloxone benzoylhydrazone were completely lost in nociceptin receptor-knockout mice, suggesting the antinociceptive effects of naloxone benzoylhydrazone are due to the antagonistic effect on the nociceptin receptor, but not the agonistic effects on opioid receptors. Taken together, our results suggest that the antagonistic activity of TRK-820 for the nociceptin receptor may be responsible, in part, for the antinociceptive effects of TRK-820.

In this study, we demonstrated that TRK-820 has a potent agonistic activity for the κ-opioid receptors, a partial agonistic activity for the μ-opioid receptor and an antagonistic activity for the nociceptin receptors. The physiological roles of nociceptin have not been fully elucidated yet and, for this purpose, specific antagonists for the nociceptin receptor are thought to be very useful. Although $[Phe^1\psi(CH_2–NH)Gly^2]$nociceptin-(1–13)–$NH_2$ was recently synthesized as a selective antagonist for the nociceptin receptor in guinea pig ileum and mouse vas deferens (Guerrini et al., 1998), several groups have reported that it acted as an agonist for the nociceptin receptor (Butour et al., 1998; Calo et al., 1998; Grisel et al., 1998; Xu et al., 1998). Although TRK-820 cannot be used as an antagonist for the nociceptin receptor because of much higher affinity for the κ-opioid receptor than for the nociceptin receptor, the finding that TRK-820 has an antagonistic activity for the nociceptin receptor indicates that TRK-820 could be a leading compound for synthesis of nonpeptidic antagonists for the nociceptin receptor.

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