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Nociceptin activation of the human ORL_1 receptor expressed in Chinese hamster ovary cells: Functional homology with opioid receptors

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Abstract

Opioid receptor-like 1 (ORL_1) receptor, a member of the superfamily of G-protein-coupled receptors has significant primary sequence homology to the μ-, δ- and κ-opioid receptors. The ORL_1 receptor is selectively activated by the recently discovered peptide nociceptin. To probe the functional homology amongst these receptors, a Chinese hamster ovary (CHO) cell line expressing the human ORL_1 receptor has been characterized. Nociceptin inhibited forskolin-stimulated increases in intracellular cAMP with an IC_{50} of 70 pM. Stimulation by nociceptin caused a 2-fold increase in the rate of [^{35}S]GTPγS binding to membranes derived from CHO cells expressing the ORL_1 receptor. Following incubation with nociceptin mitogen-activated protein kinase activity was increased by 2-fold in cells expressing the ORL_1 receptor. In non-transfected CHO cells, nociceptin had no effect on cAMP accumulation, the rate of [^{35}S]GTPγS binding or mitogen-activated protein kinase activity. Human ORL_1 receptors expressed in CHO cells selectively bound [^{125}I][Tyr^{14}]nociceptin with a K_d of 2.1 pM and a B_{max} of 2.6 pmol/mg protein. Similar to opioid receptors, nociceptin binding to the ORL_1 receptor was altered by Na^+, GTPγS and dithiothreitol. Na^+ increased the K_d of nociceptin binding to the ORL_1 receptor. GTPγS decreased the apparent B_{max} of [^{125}I][Tyr^{14}]nociceptin binding but had no effect on the K_d of the remaining sites. Pretreatment with dithiothreitol inhibited nociceptin binding to the ORL_1 receptor. Nociceptin binding was insensitive to low nanomolar concentrations of opioid receptor-selective agonists and antagonists. However, high micromolar levels of opioid receptor-selective agents inhibited the binding. Morphine, naloxone, naltrindole, *nor*-Binaltorphimine and CTAP (D-Phe–Cys–Tyr-D-Trp–Arg–Thr–Pen–Thr–NH_2) inhibited nociceptin binding to ORL_1 receptor with K_i values of 36, 24, 0.4, 8 and 28 μM, respectively. These results imply that ORL_1 is a G-protein-coupled receptor with functional as well as structural homology to opioid receptors. In addition, opioid receptor ligands may serve as starting templates for the development of ORL_1 specific ligands. © 1997 Elsevier Science B.V.

Keywords: ORL_1 receptor; Nociceptin; Orphanin FQ; MAP (mitogen-activated protein) kinase

1. Introduction

Opioid receptor-like 1 (ORL_1) receptor is a member of the G-protein-coupled receptor family with approximately fifty percent primary sequence identity to the μ-, δ- or κ-opioid receptors (Mollereau et al., 1994; Fukuda et al., 1994; Chen et al., 1994; Wang et al., 1994; Bunzow et al., 1994). This receptor was discovered through the use of cloning strategies designed to identify novel members of the opioid receptor family. Although ORL_1 has a high primary sequence homology to the opioid receptors, classical opioid receptor agonists and endorphins fail to activate the cloned ORL_1 receptor (Fukuda et al., 1994; Wang et al., 1994). Two groups have independently isolated the endogenous agonist peptide for ORL_1 receptor (Meunier et al., 1995; Reinscheid et al., 1995). The peptide has been named nociceptin by Meunier et al. (1995) and orphanin FQ by Reinscheid et al. (1995). In this report we will use the name nociceptin. Nociceptin is a seventeen amino acid peptide that displays significant sequence homology to the endogenous opioid receptor agonist dynorphin A.

The ORL_1 receptor and nociceptin colocalize with the opioid receptors in areas of the brain and the spinal cord known to be associated with neuronal pathways of pain transmission (Anton et al., 1996). In contrast to the opioid receptors, earlier reports showed that activation of the ORL_1 receptor by nociceptin leads to hyperalgesia (Meunier et al., 1995; Reinscheid et al., 1995). Recently, Tian et al. (1997) reported that intracerebroventricular administration of nociceptin has no effect on basal tail-flick

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latency but antagonizes morphine analgesia. Tian et al. (1997) also reported that intrathecal administration of nociceptin produces analgesia and potentiates the analgesic effect of morphine. Yamamoto et al. (1997) reported that intrathecally administered nociceptin attenuated the level of thermal hyperalgesia after unilateral constriction injury to the sciatic nerve in the rat. While, Dawson-Basoa and Gintzler (1997) reported that intrathecal administration of nociceptin abolished gestational and sex steroid-induced increment in jump thresholds and produced a significant hyperalgesia in the rat. Florin et al. (1996) reported that intracerebroventricular administration of nociceptin stimulates locomotion and exploratory behavior in mice. However, Nishi et al. (1997) reported that in knockout mice lacking the ORL_1 receptor nociceptive threshold and locomotor activity was not significantly different from control mice and the nociceptin system appears to participate in the regulation of the auditory system.

It is clear from the conflicting results obtained from different laboratories that the evaluation of nociceptin's central and peripheral effects is hindered by the lack of nonpeptidic small molecule selective agonist and antagonists for the ORL_1 receptor. Thus, the development of nonpeptidic selective agonists and antagonists for the ORL_1 receptor is the key for a full evaluation of nociceptin's physiological roles and its utility for therapeutic interventions. With respect to the discovery of ORL_1 receptor agonists and antagonists, the high degree of structural homology between the ORL_1 and opioid receptors poses several important issues. This high degree of homology suggests that non-peptidic opioid receptor agonists and antagonists may bind the ORL_1 receptor and could serve as structural leads for the design of potent and selective ORL_1 receptor agonists and antagonists. This report details the characterization of a Chinese Hamster Ovary (CHO) cell line expressing the human ORL_1 receptor. Results reported herein confirm that the ORL_1 receptor shares a high degree of functional homology with other members of the opioid receptor family as evidenced by the ability of nociceptin to stimulate mitogen-activated protein kinase activity. These data suggest that nonpeptidic opioid receptor agonists and antagonists could be used for the development of ORL_1 receptor selective agents.

2. Materials and methods

2.1. Cloning human ORL_1 receptor

Human ORL_1 mRNA was prepared by reverse transcription of human brain poly$(A)^+$ RNA (Clontech) using a 20-mer lower primer positioned 191 bp downstream from the stop codon. Amplification of ORL_1 cDNA was performed using an 18-mer upper primer located 29 bp upstream from the translation start codon and the same lower primer used in the reverse transcription reaction. A polymerase chain reaction (PCR) product of the expected size (1332 bp) was ligated with the eukaryotic expression vector pCR3 (Invitrogen) and transformed into competent Top10F′ cells (One Shot; Invitrogen). The resulting plasmid was named ORL_1-pCR3. The sequence of the plasmid encoding ORL_1 was obtained by automated DNA sequencing using a Perkin-Elmer 373A sequencer. The protein encoded by this clone is identical to that published previously (Mollereau et al., 1994; Fukuda et al., 1994; Chen et al., 1994; Wang et al., 1994; Bunzow et al., 1994).

2.2. Isolation of CHO cells expressing the ORL_1 receptor

CHO cells (1.5×10^7 cells) were transfected by electroporation in Krebs-Ringers buffer (120 mM NaCl, 4.6 mM KCl, 0.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.6 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, 15 mM $NaHCO_3$ and 10 mM D-glucose) with 20 μg of ORL_1-pCR3 plasmid DNA at 290 V and 906 μF in a 0.4 cm cuvette. Cells were diluted in Dulbecco's Modified Eagle's Medium Media (Life Technologies) containing 10% fetal calf serum, 1% non-essential amino acids and 1% penicillin/streptomycin. After 48 h the medium was replaced with selection medium containing 700 μg/ml active G418. G418 resistant CHO cell clones were expanded and screened for an inhibitory effect of 100 nM nociceptin on forskolin-stimulated cellular cAMP production. Active colonies were further screened using [^{125}I][Tyr^{14}]nociceptin binding assays. The clone which displayed the greatest nociceptin-mediated inhibition of forskolin-stimulated cAMP had the greatest specific activity in the binding assay. These cells were subsequently used for the studies described.

2.3. Cell culture

CHO cells were grown to 95% confluency in F-12 media (Life Technologies) plus 10% fetal calf serum, 1% non-essential amino acids, 1% L-glutamine, 200 μg/ml G-418 and 1% penicillin/streptomycin (Life Technologies). Cells were harvested by washing the monolayer once with phosphate-buffered saline (calcium and magnesium free) followed by the addition of enzyme-free cell dissociation buffer (Sigma). Cells were rinsed once in Media F-12 and resuspended in Media F-12 (without bovine serum albumin) at a final cell density of 500 000 cells/ml.

2.4. Preparation of CHO cell membranes

Confluent CHO cells expressing the receptor were dissociated from the surface of cell culture flasks using cell dissociation buffer (Sigma). Cells were pelleted at $2000 \times g$ for 15 min and the supernatant was discarded. CHO cells were resuspended in buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$ and 100 μM Pefabloc (Boehringer-Mannheim). Following 15 min on ice, the cell suspension was homogenized in a Dounce glass hand homog-

enizer. The homogenate was centrifuged at 100 000 × *g* for 60 min to pellet cell membranes. The supernatant was discarded and the pellet resuspended in buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$, 100 μM Pefabloc, 125 mM sucrose and 10% glycerol. The membrane suspension was aliquoted into small aliquots, rapidly frozen in liquid nitrogen, and stored at −80°C. Protein concentrations were determined using the micro bicinchoninic acid assay (Pierce) with bovine serum albumin as a standard.

2.5. Nociceptin binding assay

CHO cell membrane preparations expressing the ORL_1 receptor (2 μg) were incubated with varying concentration of [^{125}I][Tyr^{14}]nociceptin (3–500 pM) in a buffer containing 50 mM HEPES (pH 7.4), 10 mM NaCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mg/ml bovine serum albumin and 0.025% bacitracin. In a number of studies assays were carried out in buffer containing 50 mM Tris–HCl (pH 7.4), 1 mg/ml bovine serum albumin and 0.025% bacitracin. Samples were incubated for 1 h at room temperature (22°C). Radiolabelled ligand bound to the membrane was harvested over GF/B filters presoaked in 0.1% polyethyleneimine using a Brandell cell harvester and washed five times with 5 ml cold distilled water. Nonspecific binding was determined in parallel by similar assays performed in the presence of 1 μM nociceptin. All assay points were performed in duplicates of total and non-specific binding. Results shown are representatives of three independent experiments.

2.6. Determination of cAMP content in CHO cells

250 μl of F-12 Media (Life Technologies) containing 125 000 cells was added to each well of a 96-well plate. Following addition of 50 μl of a 670 nM nociceptin stock solution (6.7 times final assay concentration) samples were mixed and incubated for 5 min at room temperature. 35 μl of 3-isobutyl-1-methylxanthine (2 mM stock) with and without 20 μM forskolin was added to each well, mixed and incubated for 10 min at room temperature. Assays were terminated by the addition of 25 μl of 350 mM HCl. Samples were then frozen at −80°C and thawed to lyse the cells. Ruptured cell suspensions were dispersed by repeated pipetting. cAMP present in the samples was quantified using an EIA cAMP assay plate or Flashplate (Amersham) following the commercial protocols.

2.7. [^{35}S]GTPγS binding to CHO cell membranes

CHO cell membranes expressing the human ORL_1 receptor (20 μg) were incubated for 30 min at room temperature with 100–300 pM [^{35}S]GTPγS in an assay mixture (500 μl) containing 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 0.25 mg/ml bacitracin, 120 mM NaCl and 1 μM GDP. Assays were terminated by rapid filtration over GF/B filters (presoaked for 30 min in 10 mM K_2HPO_4) and washed five times with 5 ml cold (4–10°C) buffer containing 20 mM Tris–HCl (pH 8.0), 20 mM $MgCl_2$ and 100 mM NaCl. Filter-bound radioactivity was quantified by scintillation counting. Nonspecific binding was determined by performing the assay in the presence of 10 μM GTPγS. For the determination of the nociceptin-stimulated increase in the binding of [^{35}S]GTPγS, membranes were preincubated with nociceptin for 60 min prior to the initiation of the assay. All assays were performed in duplicates.

2.8. Measurement of mitogen-activated protein (MAP) kinase activity

CHO cells (wild type or cells expressing the ORL_1 receptor) were split into 6-well culture plates and allowed to grow to confluency in F12 media containing 10% fetal bovine serum and 50 μg/ml gentamicin at 37°C. Cells were serum starved overnight (F12 containing 0.5% fetal bovine serum) and stimulated for 5 min as described in the legend for Fig. 7 for 5 min. The plates were immediately placed on ice and the cells lysed by the addition of 200 μl RIPA buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 5 mM EDTA, 1% v/v Igepal, 0.5% w/v deoxycholate, 0.1% w/v sodium dodecyl sulfate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate). Cell lysates were centrifuged (15 000 × *g*, 15 min, 4°C) and supernates were transferred to 1.5 ml microfuge tubes. MAP kinase activity in each sample was assessed using a BIOTRAK p42/p44 MAP kinase enzyme assay system (Amersham). Briefly, 15 μl of the cleared cell lysate was combined with 10 μl of the specific MAP kinase substrate supplied in the kit and 5 μl of ATP/$MgCl_2$ solution containing 0.2 mCi/ml [γ-^{32}P]ATP. Assay mixture was incubated for 30 min at 30°C, and 10 ml of the supplied stop solution was added. This mixture was blotted onto binding paper and washed twice with 1.0% acetic acid and twice with water. The amount of radioactivity remaining on the binding paper was used as an index of MAP kinase activity present in the cell lysate sample.

2.9. Materials

[^{125}I][Tyr^{14}]nociceptin was obtained from NEN-DuPont and Amersham. Radioiodination of [Tyr^{14}]nociceptin was performed by the chloramine-T method and was purified by reverse phase high-performance liquid chromatography on a C_{18} column.

3. Results

3.1. Characteristics of nociceptin binding to human ORL_1 receptor

CHO cell membranes prepared from cells stably transfected with ORL_1-pCR3 show a time dependent increase

in [^{125}I][Tyr^{14}]nociceptin binding, reaching equilibrium after 60–90 min at room temperature. Under identical conditions, no specific [^{125}I][Tyr^{14}]nociceptin binding was detected in membranes prepared from non-transfected CHO cells (data not shown). [^{125}I][Tyr^{14}]nociceptin dissociation from the receptor was not significant following 60 min from dilution of the membranes into HEPES buffer. Scatchard analysis of the data derived from saturation binding studies suggests the presence of a single population of binding sites (Fig. 1). The K_d and B_{max} of [^{125}I][Tyr^{14}]nociceptin binding to the receptor were dependent on buffer composition. In Tris buffer containing no additional salts, the K_d was 2.1 pM and the calculated B_{max} was 2.3 pmol/mg protein (Fig. 1A). In HEPES buffer containing salts, the K_d was 22.2 pM and the calculated B_{max} was 1.9 pmol/mg protein (Fig. 1A). This difference in the binding characteristics was found to be a result of NaCl and other salts in the buffer. The addition of NaCl to the Tris buffer at concentrations greater than 5 mM resulted in a concentration-dependent inhibition of nociceptin binding (Fig. 2A). This effect was not limited to NaCl, as KCl, LiCl and NH_4Cl also caused a concentration-dependent inhibition of nociceptin binding (Fig. 2A). Using sulfate salts of Na^+, K^+ and NH_4^+ also caused a

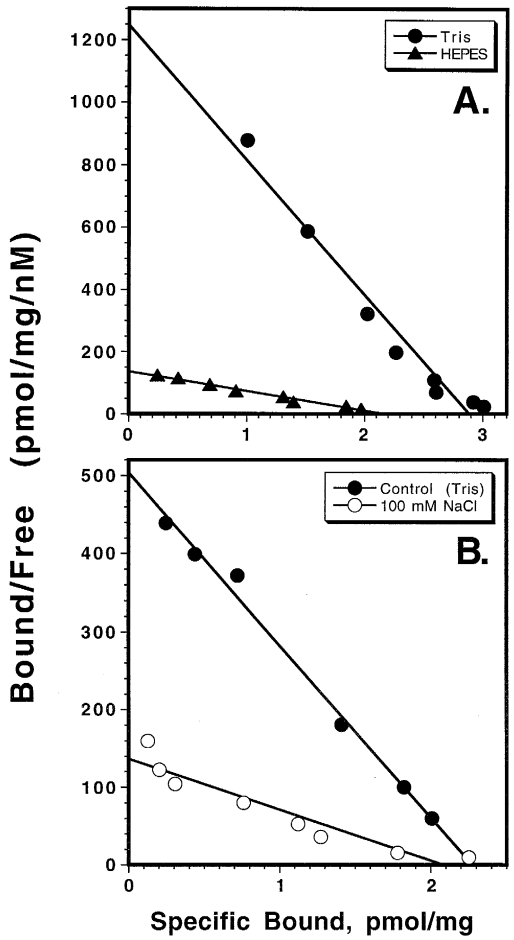

Fig. 1. Scatchard plots of [^{125}I][Tyr^{14}]nociceptin binding to human ORL_1 receptor transfected CHO cell membranes. (A) Saturation binding assays were performed using Tris–HCl (●, pH 7.4) or HEPES buffers (▲, pH 7.4) as described in Section 2. Binding assays were performed in a 2.5 ml assay volume using 2 μg membrane protein and incubated for 1 h at room temperature. The K_d and B_{max} values were derived from Scatchard analysis (Scatchard, 1949) using linear regression analysis to fit the data. (B) Scatchard plots of [^{125}I][Tyr^{14}]nociceptin binding to human ORL_1 receptor in the absence (●) and presence of 100 mM NaCl (○). Saturation binding assays were performed in Tris–HCl buffer (pH 7.4) as described above.

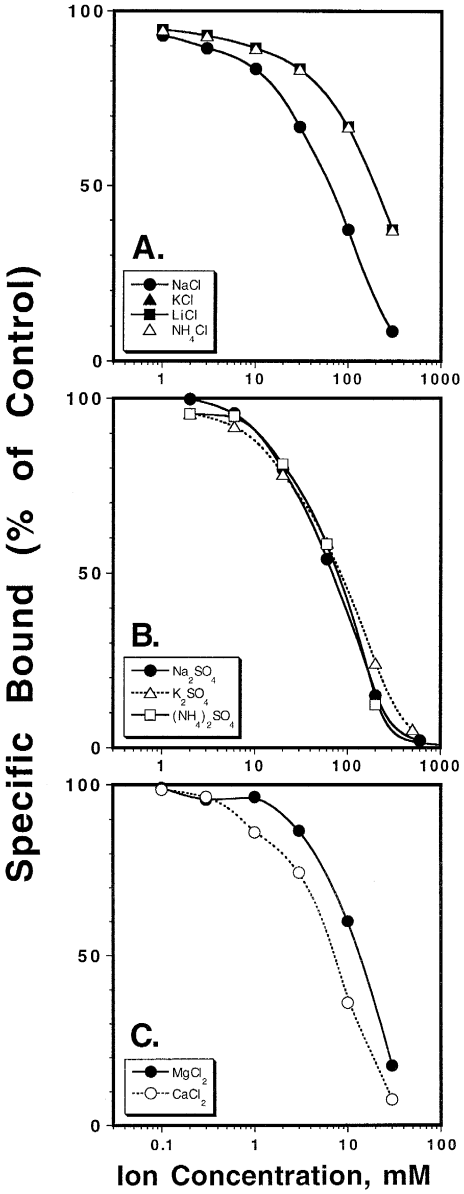

Fig. 2. Inhibition of [^{125}I][Tyr^{14}]nociceptin binding to human ORL_1 receptor by monovalent and divalent cations. Binding assays were performed in Tris–HCl buffer as described in the legend to Fig. 1. Assays were carried out in the absence and presence of varying concentrations of (A) chloride salts of Na^+ (●), K^+ (▲), Li^+ (■) and NH_4^+ (△), (B) sulfate salts of Na^+ (●), K^+ (△) and NH_4^+ (□), or (C) $MgCl_2$ (●) and $CaCl_2$ (○). Results are shown as percentage of control binding in the absence of salts.

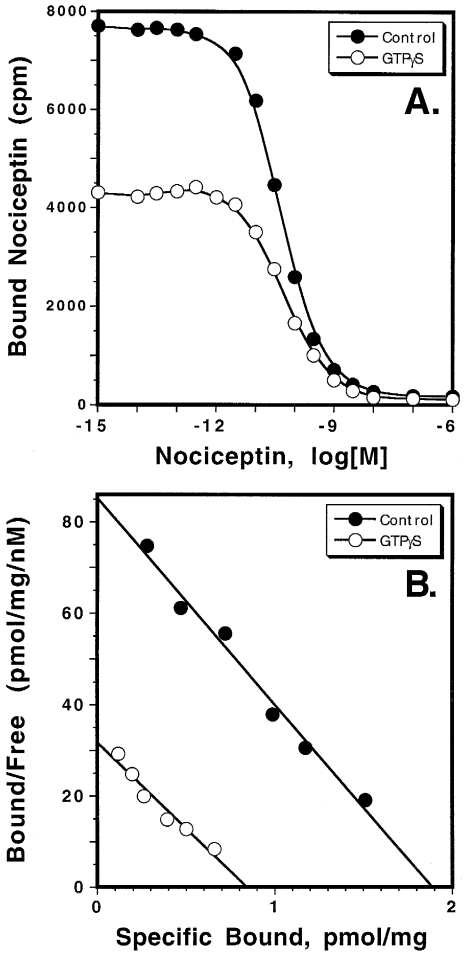

Fig. 3. Effect of GTPγS on nociceptin binding to the ORL_1 receptor. (A) The ability of nociceptin to compete with [^{125}I][Tyr^{14}]nociceptin binding to the human ORL_1 receptor was determined in the presence (○) and absence (●) of 100 μM GTPγS. Binding assays were performed in 2.5 ml assay volume (Tris buffer with 1 mM $MgCl_2$) using 2 μg membrane protein and incubated for 1 h at room temperature as described in Section 2. Membranes were preincubated 10 min with or without GTPγS prior to initiation of the binding assays. (B) Scatchard analysis of [^{125}I][Tyr^{14}]nociceptin binding to the human ORL_1 receptor. Binding assays were performed in HEPES buffer in the absence (●) and presence of 30 μM GTPγS (○) as described above.

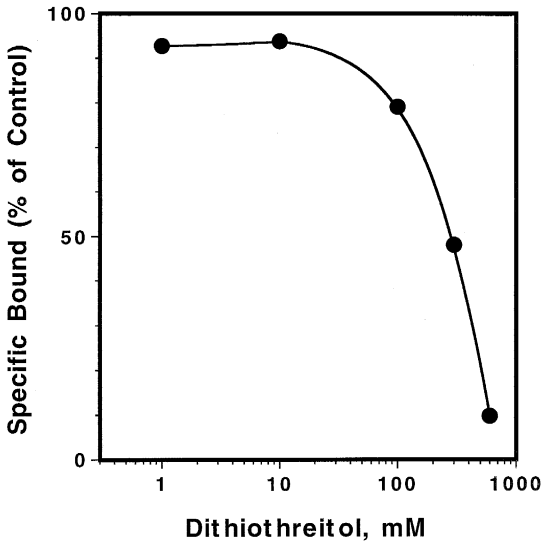

Fig. 4. Inhibition of [^{125}I][Tyr^{14}]nociceptin binding to the human ORL_1 receptor by dithiothreitol. CHO cell membranes were preincubated with varying concentrations of dithiothreitol for 5 min at room temperature prior to the initiation of binding assays. Binding assays were performed in Tris–HCl buffer (pH 7.4).

similar profile of concentration-dependent inhibition of the binding (Fig. 2B). In addition, the divalent cations Mg^{2+} and Ca^{2+} both showed a concentration-dependent inhibition of nociceptin binding to the ORL_1 receptor (Fig. 2C). As shown in Fig. 1B, 100 mM NaCl caused a sharp decrease in the affinity of nociceptin binding to the receptor with the K_d increasing to 60 pM.

As shown in Fig. 3A, addition of 100 μM GTPγS, a nonhydrolyzable GTP analogue, to the binding assay mixture reduced the apparent specific [^{125}I][Tyr^{14}]nociceptin binding to the receptor but was without effect on the IC_{50}. In saturation studies, GTPγS caused a sharp decrease in the level of detectable binding sites without affecting the apparent K_d of the remaining sites (Fig. 3B). The addition of high concentrations of dithiothreitol (> 10 mM) to the assay mixture caused a decrease in [^{125}I][Tyr^{14}]nociceptin binding to the ORL_1 receptor (Fig. 4).

[^{125}I][Tyr^{14}]nociceptin binding to the receptor was inhibited in a concentration dependent fashion by micromolar concentrations of opioid selective agonists and antagonists (Table 1). Naltrindole inhibited nociceptin binding with a K_i of 0.4 μM, while naloxone showed a K_i of 24 μM (Table 1). β-endorphin inhibited the binding at concentrations > 10 μM, while dynorphin A had a greater potency for inhibition of the binding with a K_i of 0.025 μM.

Table 1
Inhibition of nociceptin binding to human ORL_1 receptor by opioid receptor selective agonists and antagonists

Substance	K_i (nM)
Morphine	35670
Naloxone	23790
Naltrindole	466
nor-Binaltorphimine	7825
CTAP (D-Phe–Cys–Tyr-D-Trp–Arg–Thr–Pen–Thr–NH_2)	28150
β-endorphin	24430
Dynorphin A	25

[^{125}I][Tyr^{14}]nociceptin binding to membranes prepared from cells stably transfected with ORL_1-pCR3 was carried out in Tris buffer as described in Section 2. Assays were performed in a 200 μl volume using varying concentrations of the opioid receptor selective agonists and antagonists in competition with [^{125}I][Tyr^{14}]nociceptin. Maximum concentrations of peptide agonists and non-peptidic agents employed were 100 and 300 μM, respectively. K_i values were derived by analysis with GraphPad Prism (GraphPad Software) using the method of Cheng and Prusoff (1973).

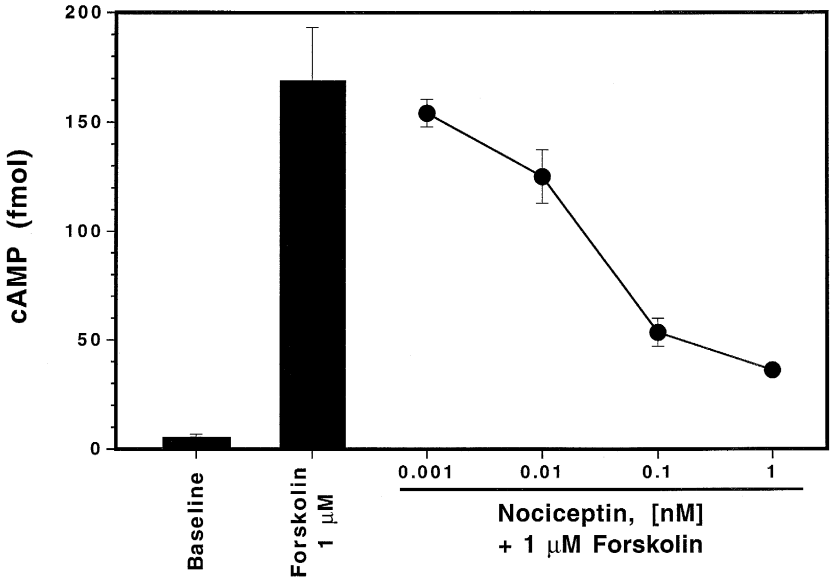

Fig. 5. Inhibition of forskolin-stimulated intracellular cAMP accumulation in CHO cells transfected with the human ORL_1 receptor by nociceptin. CHO cells were preincubated with nociceptin for 5 min prior to the addition of forskolin and IBMX. Assays and cAMP measurements were performed as described in Section 2.

3.2. Functional activation of ORL_1 receptor by nociceptin

In CHO cells expressing the ORL_1 receptor nociceptin caused a robust concentration-dependent decrease in the level of intracellular cAMP accumulation induced by 1 μM forskolin, with an EC_{50} of 70 pM and a maximal inhibition of 85% (Fig. 5). In addition, nociceptin also caused a concentration-dependent two-fold stimulation of [^{35}S]GTPγS binding to membranes prepared from these

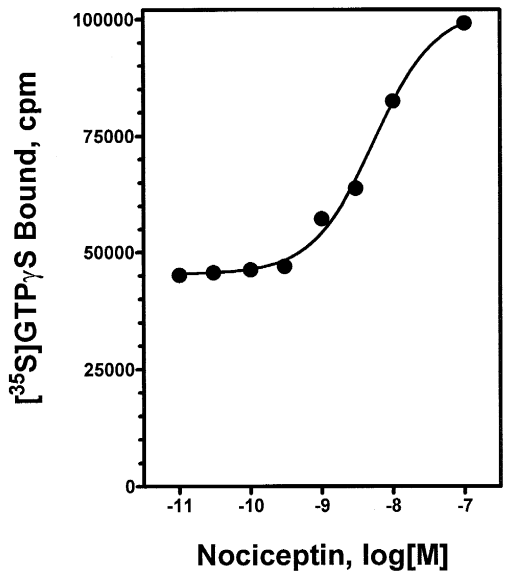

Fig. 6. Nociceptin stimulation of [^{35}S]GTPγS binding to human ORL_1 transfected CHO cell membranes. GTPγS binding assays were performed as described in Section 2.

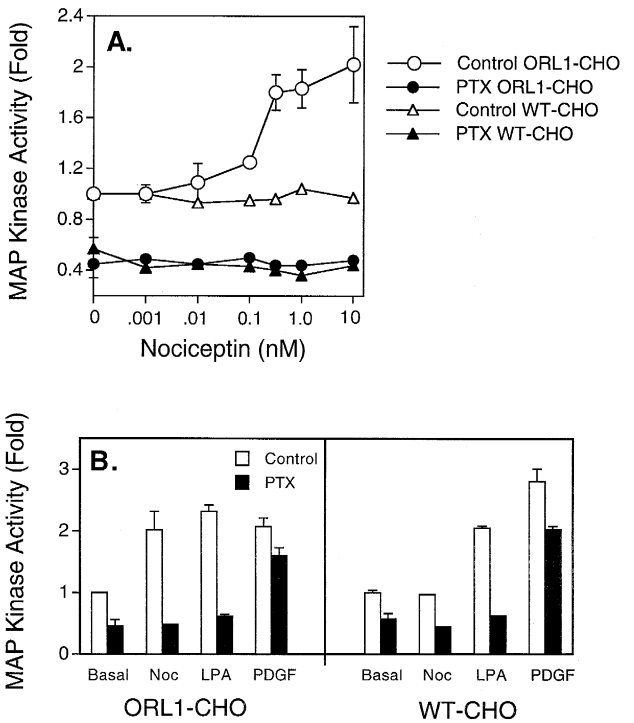

Fig. 7. Nociceptin stimulates pertussis toxin-sensitive MAP kinase activation. Wild type CHO cells (WT-CHO) and CHO cells expressing ORL_1 receptors (ORL1-CHO) were pretreated overnight with 100 ng/ml pertussis toxin (PTX, filled symbols and solid bars) or vehicle (Control, open symbols and open bars) in F12 media containing 0.5% fetal bovine serum. Cells were then stimulated for 5 min with (A) the indicated concentration of nociceptin or (B) vehicle (Basal), 10 nM nociceptin (Noc), 10 mM lysophosphatidic acid (LPA), or 10 ng/ml platelet-derived growth factor (PDGF). MAP kinase activity was then determined. Values are the means from one experiment performed in duplicate. Similar results were observed in four separate experiments.

cells (Fig. 6). Nociceptin inhibition of cAMP production suggests that the ORL_1 receptor is coupled to a G_i-like protein. Several receptors that can couple to G_i, including the opioid receptors, have been shown to activate MAP kinase (Fukuda et al., 1996; Ling-Yuan and Chang, 1996; Van Biesen et al., 1996). As shown in Fig. 7A, nociceptin stimulates the activity of MAP kinase in a concentration-dependent manner in CHO cells expressing ORL_1 receptors but not in wild type CHO cells. Pertussis toxin, which ADP-ribosylates G_i and G_o proteins, preventing receptor/G-protein coupling, inhibits nociceptin-stimulated MAP kinase activation. In Fig. 7B, the effect of pertussis toxin on nociceptin-stimulated MAP kinase activation is compared to its effect on MAP kinase activation by lysophosphatidic acid and platelet-derived growth factor (PDGF). Lysophosphatidic acid and PDGF activate MAP kinase through a G_i-coupled pathway and a tyrosine kinase receptor pathway, respectively. In ORL_1 expressing cells, pertussis toxin inhibits nociceptin and lysophosphatidic acid stimulated MAP kinase activation in a similar manner. In contrast, PDGF-stimulated MAP kinase activation, which is mediated by a G-protein independent pathway, is unaffected by pertussis toxin pretreatment. The results of Fig. 7 therefore demonstrate that ORL_1 is capable of coupling to a G_i/G_o protein to mediate MAP kinase activation.

4. Discussion

The ORL_1 receptor is a member of the superfamily of G-protein-coupled receptors and has approximately 50% homology to the μ-, δ- and κ-opioid receptors (Mollereau et al., 1994; Fukuda et al., 1994; Chen et al., 1994; Wang et al., 1994; Bunzow et al., 1994). This degree of sequence homology is similar to that seen between receptor subtypes and suggests the potential for functional homology among these receptors. Nociceptin, an endogenous ligand for ORL_1 receptor, is a 17-amino acid peptide with a high degree of homology to the opioid peptide dynorphin A (Meunier et al., 1995; Reinscheid et al., 1995). Similar to the opioid stimulation of opioid receptors, it has been shown that nociceptin stimulation of the ORL_1 receptor results in inhibition of forskolin-stimulated increases in cAMP accumulation (Meunier et al., 1995; Reinscheid et al., 1995), inhibition of calcium currents (Connor et al., 1996), and increases potassium conductance (Vaughan and Christie, 1996). In contrast to the well-known analgesic effects of opioid receptor agonists, nociceptin stimulation of the ORL_1 receptor can result in hyperalgesia (Meunier et al., 1995; Reinscheid et al., 1995) and in an inhibition of the analgesia that is produced by opioid receptor agonists (Tian et al., 1997). The ORL_1 and opioid receptors reside on distinct neurons in brain and spinal cord regions involved in pain transmission (Anton et al., 1996; Riedl et al., 1996). The mechanisms underlying these differential effects remain to be elucidated. In this study we have evaluated the functional significance of the structural similarities between the opioid and ORL_1 receptors.

The affinity of ligands for opioid receptors is altered in the presence of physiological concentrations of sodium. Generally, in the presence of sodium agonist affinities decrease and antagonist affinities increase (Simon et al., 1973; Pert et al., 1973; Werling et al., 1984, 1986; Puttfarcken et al., 1986). Analogous to the opioid receptors, nociceptin binding to the ORL_1 receptor shows a strong sodium sensitivity (Figs. 1 and 2). Other monovalent cations, e.g., K^+, Li^+ and NH_4^+, produced a similar effect on nociceptin binding (Fig. 2). In contrast to the opioid receptors (Pasternak et al., 1975), Mg^{2+} and Ca^{2+} also caused a concentration-dependent inhibition of nociceptin binding to the ORL_1 receptor. Recently, Butour et al. (1997) and Ardati et al. (1997) have also reported sodium modulation of nociceptin binding to the ORL_1 receptor. Sodium modulates the affinity of many G-protein-coupled receptor ligands, including those for the α_2-adrenoceptor (Limbird et al., 1982; Mooney et al., 1982), β-adrenoceptor (Minuth and Jakobs, 1986), dopamine D_2 (Neve et al., 1990), muscarinic (Rosenberger et al., 1980) and somatostatin receptor (Enjalbert et al., 1983). These receptors contain a conserved aspartate residue in their second transmembrane spanning region. Site directed mutagenesis of the aspartate residue to asparagine in the α_2-adrenoceptor (Horstman et al., 1990), δ-opioid receptor (Kong et al., 1993a) and the somatostatin type 2 receptor (Kong et al., 1993b) results in mutant receptors that are insensitive to the effects of Na^+. By analogy, we speculate that aspartate 97 in the second transmembrane domain of the ORL_1 receptor may also be critical for the cationic regulation of nociceptin binding.

Agonist binding to μ- and δ-opioid receptors is inhibited by millimolar concentrations of dithiothreitol (Gioannini et al., 1989; Shahrestanifar et al., 1996). A similar effect of dithiothreitol is seen on the ORL_1 receptor, with a sharp decrease in [^{125}I][Tyr^{14}]nociceptin binding observed at dithiothreitol concentrations exceeding 30 mM. Analogous to the opioid receptors, these data suggest the presence of an intramolecular disulfide bond that is required for the maintenance of an active receptor conformation. An intramolecular disulfide bond is thought to exist between two cysteine residues in the extracellular loops of G-protein coupled receptors (Dohlman et al., 1990; Dixon et al., 1987; Karnik et al., 1988).

The human ORL_1 receptor expressed in CHO cells is functionally coupled to decreases in cAMP levels (Meunier et al., 1995; Reinscheid et al., 1995; Fig. 5). The binding of nociceptin to the ORL_1 receptor is modulated by the GTP analogue GTPγS. Incubation of membranes with GTPγS results in a decrease in the apparent number of binding sites for [^{125}I][Tyr^{14}]nociceptin with no alteration in the affinity of the remaining sites (Fig. 3). These data imply that incubation with GTPγS converts about half of

the receptors to a state with an affinity too low to be detected by [^{125}I][Tyr^{14}]nociceptin in an equilibrium binding assay. Recent reports by Butour et al. (1997) and Ardati et al. (1997) also show allosteric alteration of nociceptin binding to the ORL_1 receptor by guanine nucleotides. The functional coupling of the ORL_1 receptor to a G-protein(s) is also manifest by stimulation of GTPγS binding to cell membranes in the presence of nociceptin (Fig. 6). Inhibition of forskolin stimulated cellular cAMP accumulation by nociceptin indicates that the ORL_1 receptor is coupled to a G_i-like protein. Activation of several receptors coupled to G_i and G_o, including opioid receptors, results in the stimulation of mitogen-activated protein (MAP) kinase activity (Howe and Marshall, 1993; Albas et al., 1993; Winitz et al., 1993; Van Biesen et al., 1996; Fukuda et al., 1996; Ling-Yuan and Chang, 1996). MAP kinase is a serine/threonine kinase that phosphorylates and activates numerous transcription factors. MAP kinase activation has been implicated in the regulation of numerous cellular processes including cell growth and proliferation. In CHO cells expressing the ORL_1 receptor, nociceptin stimulates MAP kinase activation in a pertussis toxin-sensitive manner (Fig. 7). Since ORL_1 is capable of coupling to G_i, as demonstrated by the ability of nociceptin to inhibit cAMP production, it is attractive to speculate that ORL_1 mediates MAP kinase activation through a G_i-dependent signaling pathway. The signaling pathway of G_i-mediated MAP kinase activation employs the $\beta\gamma$-subunit of G_i, a phosphatidylinositol-3-kinase, Src, Shc, Grb2, SOS, p21^{ras}, Raf and mitogen activated kinase/erk kinase. The potential roles of these signaling intermediates in nociceptin-stimulated MAP kinase activation have yet to be explored.

Taken together these data suggest that the signal transduction pathways activated by the opioids and nociceptin are similar and do not form the basis for the opposing effects of these agonists observed in vivo. These data have particular relevance with regard to the anti-opioid effects of nociceptin (Tian et al., 1997) and suggest that the ligands act on different cell populations rather than different signaling pathways within the same cell. That hypothesis is supported by recent data suggesting the peptides nociceptin and dynorphin, the endogenous ligands for the ORL_1 and κ-opioid receptors, respectively, are found on distinct neural circuits in the spinal cord (Riedl et al., 1996). Receptor localization and the mechanism by which these signals are integrated remain to be elucidated.

Initial reports indicated that the ORL_1 receptor was insensitive to the opioid peptides (Wang et al., 1994) although the ORL_1 receptor could be activated by micromolar concentrations of the nonspecific opioid receptor ligand etorphine (Mollereau et al., 1994). The endogenous opioid dynorphin A and its analogues bind the ORL_1 receptor with greater affinity than β-endorphin (Table 1; Meng et al., 1996; Mollereau et al., 1996) demonstrating that the endogenous opioids display selectivity in ORL_1 binding. Among the opioid receptor selective non-peptide ligands, naltrindole, a δ-opioid receptor selective antagonist (Portoghese et al., 1988), has a K_i of 466 nM for the ORL_1 receptor. A recent report by Meng et al. (1996) shows a K_i of 640 nM for naltrindole binding to the ORL_1 receptor expressed in COS 1 cells. In a recent study by Butour et al. (1997), the opioid receptor agonists Lofentanil and Etorphine were reported to inhibit nociceptin binding and forskolin-induced increase in cAMP in recombinant CHO cells expressing the ORL_1 receptor. When compared to peptide ligands, nonpeptidic ligands may utilize distinct binding sites of the receptor for binding. These data suggest that the opioid receptor ligands can serve as templates for the design of selective non-peptidic ORL_1 receptor ligands.

In summary, these data suggest that the signal transduction pathways activated by nociceptin are similar to that stimulated by the opioids and do not form the basis for their opposing effects observed in vivo. Additionally, these data suggest that non-peptidic opioid receptor agonists and antagonists may serve as lead structures for the discovery of ORL_1 receptor agonists and antagonists. The development of selective and potent ORL_1 receptor antagonists will hasten description of the physiological pathways modulated by nociceptin.

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