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Agonistic Effect of Buprenorphine in a Nociceptin/OFQ Receptor-Triggered Reporter Gene Assay

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ABSTRACT

The role of the opioid-like receptor 1 (ORL1) and its endogenous ligand, nociceptin/orphanin FQ (N/OFQ), in nociception, anxiety, and learning remains to be defined. To allow the rapid identification of agonists and antagonists, a reporter gene assay has been established in which the ORL1 receptor is functionally linked to the cyclic AMP-dependent expression of luciferase. N/OFQ and N/OFQ$_{1\text{-}13}$NH$_2$ inhibited the forskolin-induced luciferase gene expression with IC$_{50}$ values of 0.81 ± 0.5 and 0.87 ± 0.16 nM, respectively. Buprenorphine was identified as a full agonist at the ORL1 receptor with an IC$_{50}$ value of 8.4 ± 2.8 nM. Fentanyl and 7-benzylidenenaltrexone displayed a weak agonistic activity. The ORL1 antagonist [Phe$^1\Psi$(CH$_2$-NH)Gly^2]N/OFQ$_{(1\text{–}13)}$NH$_2$ clearly behaved as an agonist in this assay with an IC$_{50}$ value of 85 ± 47 nM. Thus, there is still a need for antagonistic tool compounds that might help to elucidate the neurophysiological role of N/OFQ.

The heptadecapeptide nociceptin/orphanin FQ (N/OFQ) was discovered as the endogenous ligand of the opioid-like receptor 1 (ORL1; Meunier et al., 1995; Reinscheid et al., 1995), which belongs to the family of opioid receptors and is abundantly present in the brain and spinal cord (Mollereau et al., 1994; Darland et al., 1998). N/OFQ is characterized by a high affinity (K_d ~ 56 pM; Ardati et al., 1997) and a high selectivity toward its receptor. Ligand-mediated receptor activation leads, like with the classic opioid receptors, to inhibition of the adenylate cyclase via coupling to $G_{\alpha i/o}$ (Meunier et al., 1995; Reinscheid et al., 1995). On the cellular level, functional similarity with the μ-, δ-, and κ-opioid receptor systems has also been shown for ORL1 with respect to the N/OFQ-mediated activation of potassium channels (Vaughan and Christie, 1996) and inhibition of L-, N-, and P/Q-type calcium channels (Connor et al., 1996; Knoflach et al., 1996). Surprisingly, N/OFQ displayed a pronociceptive, hyperalgesic activity in different animal pain models after i.c.v. application (Meunier et al., 1995; Reinscheid et al., 1995; Hara et al., 1997). These findings have been explained as an inhibition of stress-induced analgesia (Mogil et al., 1996a,b).

However, antinociceptive effects of N/OFQ have been reported, like the reduction in wind-up (Stanfa et al., 1996), the modulation of *N*-methyl-D-aspartate-evoked responses of neurons from the trigeminal system (Wang et al., 1996), and the inhibition of release of calcitonin gene-related peptide from sensory fibers (Helyes et al., 1997). Recent data indicate that N/OFQ attenuates thermal hyperalgesia in an animal model of neuropathic pain (sciatic nerve ligation) and displays analgesic activity after intrathecal application in the rat formalin test (Yamamota and Nozaki-Taguchi, 1997). Analysis of N/OFQ applied i.c.v. via cannulas in a rat tail-flick model also revealed an antinociceptive effect of this peptide (Rossi et al., 1998).

Thus, with respect to nociception and analgesia, the role of N/OFQ is not completely resolved, which might be at least partially due to the lack of a selective and nonpeptidic ORL1 receptor antagonist. To establish a screening system that is based on the direct identification of ORL1 receptor agonists and antagonists, a reporter gene assay has been developed using Chinese hamster ovary (CHO-K1) cells, which harbor a cyclic AMP (cAMP)-sensitive luciferase gene and the human ORL1 receptor cDNA under control of a constitutive promoter. This test system has been validated with N/OFQ showing nanomolar potency and full agonism and tested with a collection of well known opioid compounds and two truncated N/OFQ derivatives. The data obtained with buprenorphine, a clinically applied opioid with both μ- and κ-opioid receptor affinity, and a putative ORL1 antagonist (Guerrini et al., 1998), [Phe$^1\Psi$(CH$_2$-NH)Gly^2]N/OFQ$_{(1\text{-}13)}$NH$_2$, are discussed here. The ORL1 reporter gene assay presented offers a convenient approach for finding new tool compounds.

Materials and Methods

Compounds. N/OFQ, ORL1 agonist N/OFQ$_{(1\text{-}13)}$NH$_2$, and the putative antagonist [Phe$^1\Psi$(CH$_2$-NH)Gly^2]N/OFQ$_{(1\text{-}13)}$NH$_2$, as well as endomorphin-2 and bovine nocistatin, were purchased from Neosystem Laboratoire (Strassbourg, France). The pseudopeptide bond of [Phe$^1\Psi$(CH$_2$-NH)Gly^2]N/OFQ$_{(1\text{-}13)}$NH$_2$ differs from natural peptide bonds by a methyl-

ABBREVIATIONS: N/OFQ, nociceptin/orphanin FQ; ORL1, opioid receptor-like 1; cAMP, cyclic AMP; CHO, Chinese hamster ovary.

ene group at the α-carbon atom of the amino-terminal phenylalanine derivative instead of a carbonyl function, which is present in natural peptide bonds and has its origin in the α-carboxyl group of conventional amino acids. Forskolin and opioid test compounds were obtained from Research Biochemicals Inc. (Deisenhofen, Germany) and Tocris (Cologne, Germany).

Reporter Gene Assay for Human ORL1 Receptor in CHO Cells. The cDNA encoding the human ORL1 receptor has been cloned from THP-1 cells, a human, monocytic cell line, using polymerase chain reaction and integrated into the plasmid pZeoSV (InVitrogen, Leek, the Netherlands) under control of the simian virus 40 (SV40) promoter. The resultant expression plasmid pZeoORL17 was transfected into CHO-K1/pSE66/K9 cells, which harbor the cAMP reporter plasmid pSE66. The reporter plasmid pSE66 was constructed on the basis of plasmid pMAM*neo*-LUC (Clontech, Palo Alto, CA) by substitution of the Rous sarcoma virus promoter with a promoter region composed of six cAMP-responsive element elements upstream of an SV40 promoter that has been introduced from the pGL2-promoter vector (Promega, Madison, WI). In addition, pSE66 carries a G418-resistance gene under control of a second SV40 promoter, which allows for selection of stable transformants. Monoclonal ORL1-transformants of CHO-K1/pSE66/K9 have been isolated in Nutrient Mixture F-12 (Ham) with glutamine (GIBCO-BRL, Weiterstadt, Germany) supplemented with 50 μg/ml G418 (GIBCO-BRL) and 200 μg/ml zeocin (InVitrogen) by limiting dilution in 96-well plates. N/OFQ-responsive clones were identified by determining the luciferase activity of approximately 20,000 cells/well (100 μl, plated overnight) stimulated for 6 h with 1 μM forskolin (Research Biochemicals Inc.) in the presence or absence of 10 μM N/OFQ. The assay of test compounds dissolved in distilled water or dimethyl sulfoxide has been performed under the same conditions. Dimethyl sulfoxide was kept to a final concentration of maximal 1% (v/v). Luciferase assays have been performed by using a commercial kit (Boehringer-Mannheim, Mannheim, Germany). Luminescence was measured as light-counts per second using a Wallac-Trilux counter (Wallac, Finland). Clone CHO-K1/pSE66/K9/pZeoORL17/K21 was finally selected for further studies with different test compounds. Tests for antagonism were carried out in the presence of 10 nM N/OFQ.

Results

Establishment of an ORL1 Receptor Gene Cell Line for Functional Analysis of Receptor Ligands. To analyze the agonistic-versus-antagonistic properties of ORL1 receptor ligands, a cell-based assay system was established in a two-step procedure. First, CHO-K1 cells were transformed with a plasmid carrying the firefly luciferase gene under control of a cAMP-inducible promoter and a G418-resistance gene for selection of stable transformants. Monoclonal transformants were tested for luciferase expression in the presence of increasing concentrations of forskolin, which is a direct activator of adenylate cyclases. All clones tested showed a concentration-dependent stimulation of luciferase expression (Fig. 1). In the second step, clone CHO-K1/pSE66/K9 was selected for transformation with pZeoORL17, a pZeoSV derivative carrying a cDNA encoding the human ORL1 receptor and a zeocin-resistance gene. Monoclonal transformants were produced and tested for an N/OFQ-mediated inhibition of forskolin-stimulated luciferase expres-

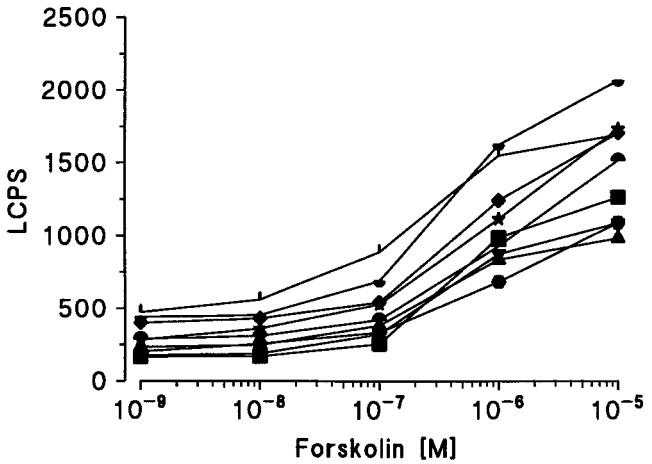

Fig. 1. Concentration-dependent expression of luciferase activity in 10 different clones of CHO-K1/pSE66 during stimulation with forskolin. Determination of luciferase-activity was carried out 20 h after the addition of forskolin. Clone 9, selected for further experiments, is marked by a filled half-circle.

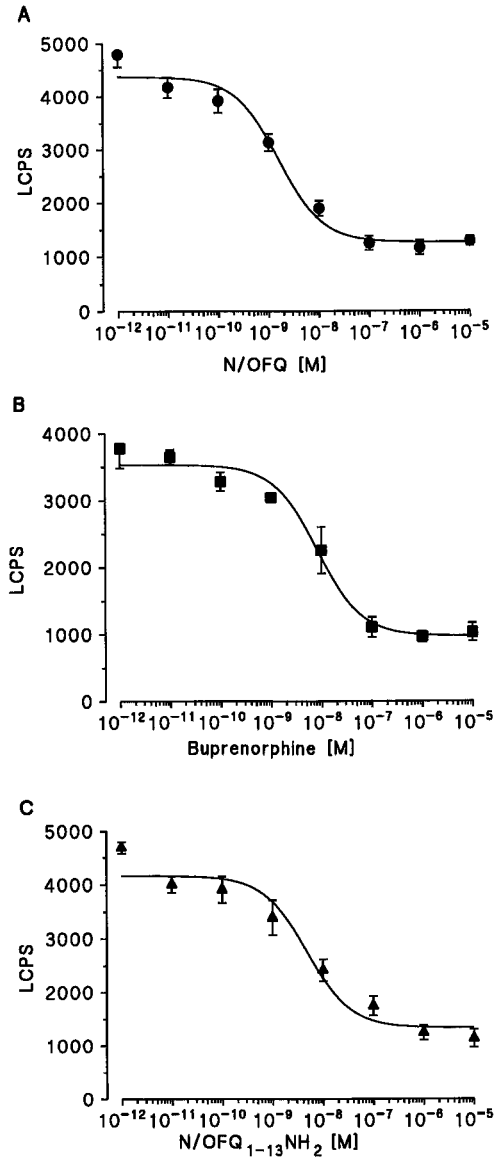

Fig. 2. Inhibition of forskolin-induced luciferase expression by activation of the recombinant ORL1-receptor using increasing concentrations of (a) N/OFQ, (b) buprenorphine, or (c) $N/OFQ_{1-13}NH_2$. Each graph represents an example of a series with three or more independent experiments. Mean values represent three data points from one representative experiment. The incubations with ORL1 agonist were performed for 6 h before cell lysis.

sion. The ORL1 receptor is coupled to $G_{\alpha i/o}$ proteins, and therefore, N/OFQ-mediated receptor activation leads to a decrease in the intracellular cAMP concentration. With the cell-based reporter gene assay, this effect is monitored by an N/OFQ-mediated inhibition of forskolin-induced luciferase expression, as shown in Fig. 2A. Using the parent cell line CKO-K1/pSE66/K9, which does not express ORL1, no inhibition of forskolin-induced luciferase-gene expression was found with N/OFQ or any other ORL1 agonist described below (data not shown). The concentration-effect curve of N/OFQ-mediated inhibition of luciferase expression is of typical sigmoidal shape. In a series of independent experiments ($N = 19$, $n = 3$; N is the number of experiments, and n is the number of parallel replications within each experiment), the mean IC_{50} value for N/OFQ in the cell-based assay was found to be 0.81 ± 0.5 nM. The mean maximal efficacy of N/OFQ observed in this test system was 81.9 ± 8.6% inhibition of cAMP-induced luciferase expression (at 10 μM N/OFQ; see Table 2).

Analysis of Opioid Reference Compounds. The selectivity of the receptor system has been assessed using a collection of opioid receptor ligands that were tested for agonistic and antagonistic activity at the ORL1 receptor (Table 1). With the exception of buprenorphine, 7-benzylidenenaltrexone, and fentanyl, none of the compounds, including dynorphin, showed an agonistic or antagonistic activity. Although 7-benzylidenenaltrexone and fentanyl showed only weak agonistic activity, buprenorphine displayed a relatively high potency with an IC_{50} value of 8.5 ± 1.5 nM ($N = 3$, $n = 3$), which is only 10-fold lower than that of N/OFQ (IC_{50} = 0.81 ± 0.5 nM). The efficacy of buprenorphine was similar to that of N/OFQ: both compounds strongly reduced the forskolin-induced luciferase expression (Fig. 2, A and B; Table 2).

Analysis of Selective N/OFQ Derivatives. To assess the agonistic-versus-antagonistic activity of $N/OFQ_{(1\text{-}13)}NH_2$ and $[Phe^1\Psi(CH_2\text{-}NH)Gly^2]N/OFQ_{(1\text{-}13)}NH_2$, respectively, both compounds were tested in the reporter gene assay. As expected, $N/OFQ_{(1\text{-}13)}NH_2$ inhibited forskolin-induced luciferase expression and showed a very similar profile as N/OFQ (Fig. 2C). The IC_{50} value of this truncated N/OFQ analog was found to be 0.87 ± 0.16 nM ($N = 3$, $n = 3$), which is highly similar to the potency value of N/OFQ itself (IC_{50} = 0.81 ± 0.5 nM). In addition, $N/OFQ_{(1\text{-}13)}NH_2$ reduced the forskolin-stimulated luciferase expression to the same level achieved by N/OFQ, which indicates that $N/OFQ_{(1\text{-}13)}NH_2$ is a full agonist. However, testing of the putative antagonist $[Phe^1\Psi(CH_2\text{-}NH)Gly^2]N/OFQ_{(1\text{-}13)}NH_2$ revealed that this compound clearly also has agonistic activity in the ORL1 reporter gene assay (Fig. 3A). The potency of $[Phe^1\Psi(CH_2\text{-}NH)Gly^2]N/OFQ_{(1\text{-}13)}NH_2$ is weaker than that of N/OFQ, $N/OFQ_{(1\text{-}13)}NH_2$, or buprenorphine, with a mean IC_{50} value of 85 ± 47 nM ($N = 3$, $n = 3$). The efficacy of $[Phe^1\Psi(CH_2\text{-}NH)Gly^2]N/OFQ_{(1\text{-}13)}NH_2$ is similar to that of the other agonists in showing strong inhibition of forskolin-stimulated luciferase-expression (maximal efficacy at 10 μM = 88.0 ± 7.5% inhibition). The application of increasing concentrations of N/OFQ in the presence of $[Phe^1\Psi(CH_2\text{-}NH)Gly^2]N/OFQ_{(1\text{-}13)}NH_2$ (between 32 nM and 3.2 μM) did not yield a rightward shift of the concentration-effect curve of N/OFQ that would be expected in case of an antagonistic activity of the pseudopeptide. Therefore, the combination experiment confirmed the agonistic property of the pseudopeptide under these test conditions (Fig. 3B).

Discussion

The ORL1 reporter gene system established in CHO-K1 cells transfected with a cAMP-sensitive luciferase gene and an ORL1 expression cassette proved to be a fast and suitable functional assay. Although even as a scintillation-proximity assay the classic radioimmunoassay for direct cAMP detection consists of a relative laborious sequence of cell extraction, centrifugation, drying, dilution, and incubation steps, including a final incubation of 15 to 20 h, the entire reporter gene assay can be performed within 7 h as a one-well reaction with only one pipetting and one vortexing step after the addition of forskolin and test compounds. In addition, due to a wide detection range of the luciferase reaction (three log scales), the assay can be performed without any dilutions of the cell lysate, in contrast to the cAMP radioimmunoassay, which covers only two log scales. Therefore, this assay format is applicable for the analysis of large compound libraries using high-throughput screening robotics. The mother cell line, CHO-K1/pSE66/K9, harboring the reporter gene alone will be useful for the establishment of functional assays for other $G_{\alpha i/o}$- or $G_{\alpha s}$-coupled receptors.

TABLE 1
Classification of opioid reference compounds
Results obtained in the ORL1 receptor reporter gene assay after testing for ORL1 agonism (test of compound alone at a final concentration of 10 μM) or ORL1 antagonism (test of compound at a final concentration of 10 μM in presence of 10 nM N/OFQ).

Receptor Selectivity	Opioid/Compound without ORL1 Agonism or Antagonism	ORL Agonist
μ	Morphine, DAMGO, levomethadone, levorphanol, naloxone, naltrexone-methobromid, tramadol, (±)-mono-*O*-demethyl-tramadol, pethidin, endomorphin-2	Fentanyl (weak), buprenorphine
δ	Deltorphin, DPDPE, pCl-DPDPE, SNC80, ICI174.864, SB213.698, naltrindole, naltribene	7-Benzylidenenaltrexone (weak)
κ	Dynorphin, bremazocine, pentazocine, nalbuphine, (−)-*trans*-(1*S*,2*S*)-U-50488, (±)-*trans*-U-50488, U-69593	
ORL1		N/OFQ, $N/OFQ_{1\text{–}13}NH_2$, $[Phe^1\Psi(CH_2\text{-}NH)Gly^2]N/OFQ_{(1\text{–}13)}NH_2$
Misc.	Nocistatin, dextromethorphan	

DAMGO, [D = Ala^2, *N* = $MePhe^4$, Gly = ol^5]enkephalin; DPDPE, [D = Pen^2, D = Pen^5]enkephalin.

The ORL1 reporter gene assay has been used for investigation of the activity of N/OFQ and a set of opioid reference compounds. N/OFQ displayed an IC_{50} value of 0.81 ± 0.5 nM, which correlates very well to the IC_{50} value of N/OFQ (1.05 ± 0.21 nM) determined in a direct cAMP assay using the cloned rat receptor LC132 (Meunier et al., 1995). However, both potency values are about 10 to 20 times higher than the K_d value of 56 pM found in the [^{3}H]N/OFQ-radioligand-binding assay (Ardati et al., 1997). This discrepancy might be explained by different factors, such as the NaCl dependence of the equilibrium between the low- and high-affinity receptor population, which is shifted toward the inactive population at higher salt concentrations (Childers and Snyder, 1980). In contrast to the radioligand-binding assay, which is performed under low salt conditions (no added NaCl), the reporter gene assay is carried out in presence of 130 mM NaCl (Ham's F-12 medium). The maximal efficacy of N/OFQ was found to be 81.9 ± 8.6% inhibition of forskolin-induced luciferase activity, which suggests that N/OFQ probably is a full agonist in this test system.

To further analyze the reporter gene assay, a set of μ-, δ-, and κ-opioid reference compounds was tested, including dynorphin and endomorphin-2, as well as nocistatin (Table 1). With the exception of buprenorphine, 7-benzylidenenaltrexone, and fentanyl, none of the compounds showed an agonistic or antagonistic activity, which underlines the selectivity of both the ORL1 receptor and the opioid receptor ligands tested. The lack of agonistic or antagonistic effects of dynorphin under the test conditions applied in this study should be discussed with respect to published data. Butour et al. (1997) reported that dynorphin binds with nanomolar affinity (K_i = 110 nM) to the ORL1 receptor but displays only a weak agonistic activity at high concentrations determined in a direct assay of cAMP (cAMP assay, IC_{50} > 10,000 nM). These data indicate that dynorphin has only a very low intrinsic efficacy at the ORL1 receptor, which probably is too low to induce an effect in the ORL1 reporter gene assay. Similarly, the lack of an antagonistic effect of dynorphin might be due to the high intrinsic efficacy of N/OFQ, which is able to raise full agonism with only very low receptor occupancy. For buprenorphine (Fig. 2B), 7-benzylidenenaltrexone, and fentanyl (Table 2), an ORL1 receptor agonism has not been described before. However, it was reported that fentanyl displays micromolar affinity to the ORL1 receptor without functional agonism (Butour et al., 1997). The discrepancy with our data might be explained by the apparently low potency and weak ORL1 agonistic efficacy of fentanyl (57% inhibition at 10 μM final concentration), which might be undetectable in other functional assays. Also, 7-benzylidenenaltrexone displayed only a weak agonistic activity at the ORL1 receptor, with 58% inhibition (10 μM final concentration).

Buprenorphine displayed a relatively high potency with an IC_{50} value of 8.5 ± 1.5 nM (N = 3, n = 3), which is only 10-fold lower than that of N/OFQ (IC_{50} = 0.81 ± 0.5 nM). The relative efficacy of buprenorphine was similar to that of N/OFQ; therefore, buprenorphine apparently is a full agonist in the ORL1 reporter gene assay. Buprenorphine is structurally related to etorphine, another opioid that is a partial agonist at the ORL1 receptor with a potency (IC_{50}) of 460 nM in a direct cAMP assay using recombinant CHO cells expressing ORL1 (Walker et al., 1995). Buprenorphine is a marketed opioid (Temgesic; F. Hoffman La Roche, Grenzach, Germany; Reckitt & Colman, Hull, UK), with affinity to μ- and κ-opioid receptors, that induces an antinociceptive effect in animal pain models (Dum and Herz, 1981) and clinically is used for the treatment of moderate-to-severe pain. In the vocalization test in the rat after electrical stimulation of the tail root, buprenorphine displayed a biphasic dose-response curve with an antinociceptive peak effect at 0.5 mg/kg and a decreased antinociceptive effect at higher or lower doses (Dum and Herz, 1981). Interestingly, buprenorphine was also found to antagonize the antinociceptive effects of several μ-opioid receptor agonists in a rhesus monkey tail withdrawal test (Walker et al., 1995). The ORL1 agonistic activity of buprenorphine found in this study may contribute to the

TABLE 2

Data obtained for ORL1 receptor agonists using the reporter gene assay

The percentage of inhibition at a agonist concentration of 10 μM was calculated using the equation (1 − [$LCPS_{ago}$ − $LCPS_{unst}$/$LCPS_{fors}$ − $LCPS_{unst}$]) × 100%, where $LCPS_{ago}$ is the forskolin-induced luciferase activity after agonist stimulation, $LCPS_{unst}$ is the background activity without forskolin, and $LCPS_{fors}$ is the forskolin-induced luciferase activity in absence of test compounds.

Ligand	*N/n*	Potency IC_{50}	Efficacy Inhibition at 10 μM
		nM	*%*
N/OFQ	19/3	0.81 ± 0.5	81.9 ± 8.6
Buprenorphine	3/3	8.4 ± 2.8	82.9 ± 2.1
Fentanyl	2/3	~10.000	57.5
7-Benzylidenenaltrexone	2/3	~10.000	58.3
$N/OFQ_{(1–13)}NH_2$	3/3	0.87 ± 0.16	87.2 ± 3.5
[$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]-$N/OFQ_{(1–13)}NH_2$	3/3	85 ± 47	88.0 ± 7.5

LCPS, light counts per second.

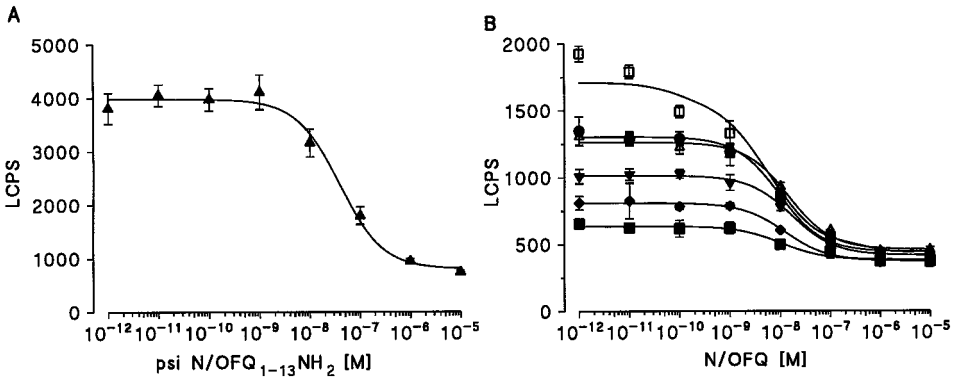

Fig. 3. A, concentration-dependent effect of [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]$N/OFQ_{(1–13)}NH_2$ on the forskolin-induced luciferase expression by activation of the recombinant ORL1 receptor. The incubations with both ligands was performed for 6 h before cell lysis. Each mean value represents three data points from one representative experiment of three experiments. B, effect of increasing concentrations of [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]$N/OFQ_{(1–13)}NH_2$ on the concentration-effect curve of N/OFQ (□). The following concentrations of the pseudopeptide were used: ●, 32 nM; ▲, 100 nM; ▼, 320 nM; ♦, 1 μM; and ■, 3.2 μM.

biphasic dose-response curve as well as to the antiopioid effects observed after the application of μ receptor agonists. It should be recalled that also for the endogenous ligand, N/OFQ, an antiopioid activity has been described after the application of standard opioids (Mogil et al., 1996a,b).

Testing the only ORL1 antagonist described so far, the truncated N/OFQ derivative [Phe$^1\Psi$(CH$_2$-NH)Gly^2]N/OFQ$_{(1\text{-}13)}$NH$_2$ (Guerrini et al., 1998), we did not observe the expected rightward shift of the N/OFQ concentration-effect curve but an agonistic activity of this peptide alone, with an IC_{50} value of 85 $\pm$ 47 nM. There also is evidence from other laboratories that the putative antagonist has agonistic properties. It was found that [Phe$^1\Psi$(CH$_2$-NH)Gly^2]N/OFQ$_{(1\text{-}13)}$NH$_2$ did not antagonize the pronociceptive and antinociceptive effects of N/OFQ after i.c.v. or i.t. application in the rat tail-flick assay (Candeletti et al., 1998). Using the guanosine-5′-*O*-(3-thio)triphosphate assay with membranes from different rat tissues, an agonistic activity of [Phe$^1\Psi$(CH$_2$-NH)Gly^2]N/OFQ$_{(1\text{-}13)}$NH$_2$ was shown in the frontal cortex, hypothalamus, and vas deferens. In addition, the compound induced food intake in rats like N/OFQ itself (Nicholson et al., 1998). Testing a recombinant CHO-K1 line expressing ORL1, Butour et al. (1998) observed a concentration-dependent reduction in intracellular cAMP with [Phe$^1\Psi$(CH$_2$-NH)Gly^2]N/OFQ$_{(1\text{-}13)}$NH$_2$. Finally, Xu et al. (1998) showed that the putative antagonist has the same efficacy and potency as N/OFQ in a flexor reflex model in the rat. The discrepancy between the initially stated ORL1 antagonism of the pseudopeptide (Guerrini et al., 1998) that has been shown in a vas deferens assay and the data on agonistic activity in several models might be due to different regulatory circuits in the periphery and the central nervous system or be due to a different effect on the intracellular effector pathways of the ORL1 receptor.

The ORL1 reporter gene assay described in this study allows a rapid screening of test compounds with respect to agonistic or antagonistic activity at the cloned human ORL1 receptor. The putative peptidic N/OFQ antagonist [Phe$^1\Psi$(CH$_2$-NH)Gly^2]N/OFQ$_{(1\text{-}13)}$NH$_2$ clearly showed an agonistic activity in this cellular test system that underlines the need for identifying or designing new antagonistic tool compounds. Buprenorphine was found to be a relatively potent ORL1 agonist in the reporter gene assay, which might contribute to the very specific pharmacological profile of this compound in vivo.

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