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Effects of [(*p*F)Phe^4]nociceptin/orphanin FQ-(1–13)NH_2 on GTPγ^{35}S binding and cAMP formation in Chinese hamster ovary cells expressing the human nociceptin/orphanin FQ receptor

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Abstract

Nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand for the N/OFQ receptor (NOP). In this study using Chinese hamster ovary (CHO) cells expressing the human NOP (CHO_{hNOP}) and GTPγ^{35}S binding and cAMP inhibition assays, we have characterised a novel N/OFQ ligand, [(*p*F)Phe^4]N/OFQ-(1–13)NH_2, ([(*p*F)Phe^4]). [(*p*F)Phe^4] was produced by insertion of a fluorine atom into the *para* position of the phenyl ring of Phe^4 of the truncated N/OFQ peptide N/OFQ-(1–13)NH_2. In CHO_{hNOP} membranes [(*p*F)Phe^4] and N/OFQ-(1–13)NH_2 stimulated GTPγ^{35}S binding with pEC_{50} (mean ± S.E.M.) values of 9.55 ± 0.01 and 8.94 ± 0.5 ($P < 0.05$), respectively. In whole CHO_{hNOP} cells [(*p*F)Phe^4] and N/OFQ-(1–13)NH_2 inhibited forskolin stimulated cAMP formation with pEC_{50} values of 10.19 ± 0.06 and 9.60 ± 0.04, respectively ($P < 0.05$). [(*p*F)Phe^4] was more potent (~ 4 fold) than N/OFQ-(1–13)NH_2. In both assays, the effects of [(*p*F)Phe^4] and N/OFQ-(1–13)NH_2 were pertussis toxin sensitive and reversed by the NOP antagonists J-113397 (pA_2/pK_B values 7.89–8.53) and III-BTD (pA_2/pK_B values 7.27–7.96). [(*p*F)Phe^4] is therefore a potent full agonist at NOP receptors that will be useful as pharmacological tool for defining the role of N/OFQ–NOP system in health and disease.

Keywords: Nociceptin/orphanin FQ; [(*p*F)Phe^4]N/OFQ-(1–13)NH_2; GTPγ^{35}S binding; cAMP formation; Recombinant, NOP receptor; NOP receptor antagonist

1. Introduction

Nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand for the opioid-like seven transmembrane spanning N/OFQ receptor, NOP (Meunier et al., 1995; Reinscheid et al., 1995). Receptor (NOP) and peptide (N/OFQ) abbreviations used in this paper are in line with recent IUPHAR recommendations (Cox et al., 2000). N/OFQ–NOP system is involved in the control of a range of physiological responses including: nociception, locomotion, feeding, memory processing, anxiety and control of the cardiovascular system (Calo' et al., 2000c; Mogil and Pasternak, 2001). At the cellular level nociceptin increases a K^+-channel conductance, inhibits voltage activated Ca^{2+}-channels and inhibits adenylyl cyclase to reduce cAMP formation via pertussis toxin sensitive $G_{i/o}$ G-proteins (Hawes et al., 2000; Calo' et al., 2000c; Mogil and Pasternak, 2001). These events combine to reduce neuronal excitability, dampen cellular transmission and NOP activation has been shown to inhibit the release of a range of transmitters in the central nervous system (Schlicker and Morari, 2000) and the periphery (Giuliani et al., 2000).

It is well established that N/OFQ-(1–13) is the smallest N/OFQ fragment retaining full activity and its potency is increased by amidation (Guerrini et al., 1997; Okawa et al., 1999; Calo' et al., 2000c). In addition, this fragment mimics the action of N/OFQ (Calo' et al., 2000a). Using N/OFQ-(1–13)NH_2 as a template, we have generated several molecules including [F/G]N/OFQ-(1–13)NH_2 which acts as a partial agonist (see Calo' et al., 2000c) and [Nphe^1]N/OFQ-(1–13)NH_2 which is a pure and competitive antagonist (Calo' et al., 2000b; Hashimoto et al., 2000). Substitu-

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tion of a Fluorine-atom into the *para* position of the phenyl ring in Phe^4 of N/OFQ-(1–13)NH_2 has been shown to increase the potency of this novel peptide (Guerrini et al., 2001). In this study, we have further characterised this molecule and compared it with the N/OFQ-(1–13)NH_2 template in a series of GTPγ^{35}S binding and cAMP assays in Chinese hamster ovary cells expressing the human NOP (CHO_{hNOP}). We confirm increased potency relative to N/OFQ-(1–13)NH_2 (in two assay systems), report pertussis toxin sensitivity and reversibility by the selective and non-selective NOP antagonists 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (J-113397, Kawamoto et al., 1999; Ozaki et al., 2000; Bigoni et al., 2000) and (3*S*,6*S*,9*R*)-2-oxo-3-amino-7-thia-1-aza-bicyclo[4.3.0]nonane-9-carboxylic acid (III-BTD, Becker et al., 1999; Hashiba et al., 2001).

2. Materials and methods

2.1. Sources of materials

N/OFQ-(1–13)NH_2, [(*p*F)Phe^4]N/OFQ-(1–13)NH_2, J-113397 were synthesised at one of our institutes (Ferrara) as described (Guerrini et al., 1997, 2001; De Risi et al., 2001). III-BTD was purchased from Neosystem (Strasbourg, France). [2,8-^{3}H]cAMP (28 Ci/mmol) and GTPγ^{35}S (1250 Ci/mmol) were from NEN DuPont (Boston, MA, USA). Pertussis toxin was from Sigma (Poole, Dorset, UK). All tissue culture media were from Invitrogen (Paisley, Scotland). All other reagents were of the highest purity available. CHO_{hNOP} cells were provided by Dr. F. Marshall and Mrs. N. Bevan of GlaxoSmithKline (Stevenage, Herts, UK).

2.2. Cell culture and membrane preparation

CHO_{hNOP} cells were maintained in Dulbecco's MEM/Nutrient F12 (50/50) supplemented with 5% foetal calf serum, penicillin (100 IU/ml) streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). Stock cultures were further supplemented with geneticin (G418) (200 μg/ml) and Hygromycin B (200 μg/ml) as described previously (Okawa et al., 1999). Cells were cultured at 37 °C in 5% carbon dioxide humidified air, sub-cultured twice weekly and used when confluent. For GTPγ^{35}S assays membranes were prepared from freshly harvested cell suspensions in Tris (50 mM), EGTA (0.2 mM) pH 7.4. These were homogenised then centrifuged at 13,500 rpm for 10 min at 4 °C, this was carried out a total of three times. Protein was assayed according to Lowry et al. (1951).

2.3. GTPγ^{35}S assay

CHO_{hNOP} membranes (20 μg) were incubated in 0.5 ml buffer containing Tris (50 mM), EGTA (0.2 mM), $MgCl_2$ (1 mM), NaCl (100 mM), bacitracin (0.15 mM), amastatin, bestatin, captopril and phosphoamidon (10 μM), GDP (100 μM) and ~150 pM GTPγ^{35}S (Berger et al., 2000; Hashiba et al., 2002). N/OFQ-(1–13)NH_2, [(*p*F)Phe^4], J-113397 and III-BTD were included in different combinations and various concentrations. Non-specific binding was obtained in the presence of 10 μM GTPγS. The reaction was incubated for 1 h at 30 °C with gentle shaking and terminated by filtration through Whatman GF/B filters using a Brandel-Harvester. In studies where pertussis toxin was included cultures were treated 20 h prior to experimentation with 100 ng pertussis toxin/ml of supplemented culture medium (Okawa et al., 1999).

2.4. Cyclic AMP assays

Whole cells were suspended in Krebs/HEPES buffer containing isobutylmethylxanthine (1 mM) and forskolin (1 μM) as described in (Okawa et al., 1999; Hashiba et al., 2002). N/OFQ-(1–13)NH_2, [(*p*F)Phe^4], J-113397 and III-BTD were included in different combinations and various concentrations. cAMP was extracted and assayed using a specific protein-binding assay (Okawa et al., 1999). In studies where pertussis toxin was included cultures were treated 20 h prior to experimentation with 100 ng pertussis toxin/ml of supplemented culture medium (Okawa et al., 1999).

2.5. Data analysis

All data are expressed as mean ± S.E.M. from a minimum of three experiments performed as single points or in duplicate. In GTPγ^{35}S binding studies data are either presented as disintegrations/min ^{35}S bound or stimulation factor (basal specific/agonist stimulated specific counts). cAMP data are presented as a percentage inhibition of the forskolin-stimulated response. Concentration–response curves and Schild regression plots were fitted using PRISM 3.0 (GraphPad, San Diego, USA), generating pEC_{50} and pA_2 values as appropriate. pEC_{50} values for [(*p*F)Phe^4], N/OFQ-(1–

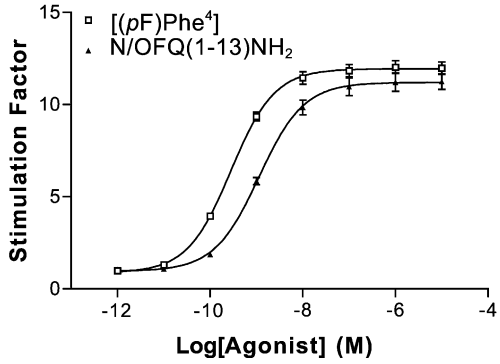

Fig. 1. N/OFQ-(1–13)NH_2 and [(*p*F)Phe^4] stimulate GTPγ^{35}S binding to CHO_{hNOP} membranes. Data (stimulation factor, i.e., ratio relative to basal) are mean ± S.E.M. ($n \geq 4$).

Table 1
[(*p*F)Phe^4] and N/OFQ-(1–13)NH_2 (10 μM) stimulated GTPγ^{35}S binding and inhibition of forskolin stimulated cAMP formation is pertussis toxin (100 ng/ml) sensitive

Assay/treatment	Peptide	
	N/OFQ-(1–13)NH_2	[(*p*F)Phe^4]
GTPγ^{35}S bound (disintegrations/min)		
Control	5981 ± 55	6480 ± 55
Pertussis toxin (100 ng/ml)	506 ± 33[a]	589 ± 9[a]
cAMP inhibition (%)		
Control	103 ± 1	102 ± 2
Pertussis toxin (100 ng/ml)	−2 ± 1[a]	−1 ± 4[a]

Data are mean ± S.E.M. (n = 3).
[a] P < 0.05 significantly different from control.

13)NH_2 and pertussis toxin sensitivity were analysed using unpaired Students' t-test. In cAMP studies, pK_B values were calculated using the formula pK_B = − log{(CR-1)/[antagonist]}, where CR is the ratio of the EC_{50} of the agonist in the presence and absence of antagonist assuming a slope value of one.

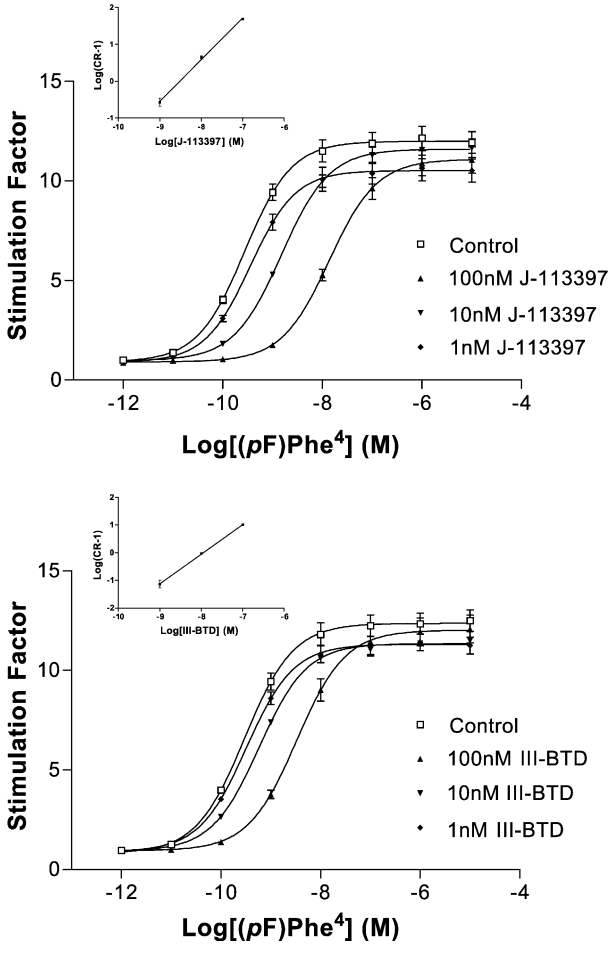

Fig. 2. Competitive antagonism of [(*p*F)Phe^4] stimulated GTPγ^{35}S binding by J-113397 (upper panel) and III-BTD (lower panel) to CHO_{hNOP} membranes. Inserts show relative Schild regression plots. Data are mean ± S.E.M. ($n \geq 4$).

Table 2
pA_2 and pK_B values for J-113397, III-BTD against [(*p*F)Phe^4] in GTPγ^{35}S and cAMP assays using CHO_{hNOP} membranes and whole cells, respectively

Antagonist	GTPγ^{35}S/pA_2	cAMP/pK_B
J-113397	8.53 ± 0.06	7.96 ± 0.05[a]
III-BTD	7.89 ± 0.17	7.27 ± 0.15[a]

Slope factors for the Schild plots in GTPγ^{35}S assays did not differ from unity.
Data are mean ± S.E.M. (n = 4–5).
[a] P < 0.05 significantly less potent than observed in the GTPγ^{35}S assay.

3. Results

3.1. GTPγ^{35}S assays

In CHO_{hNOP} membranes [(*p*F)Phe^4] stimulated GTPγ^{35}S binding in a concentration-dependent and saturable manner; pEC_{50} 9.55 ± 0.01 and E_{max} 11.94 ± 0.30 (n = 11) (Fig. 1). In the same preparation N/OFQ-(1–13)NH_2 stimulated GTPγ^{35}S binding with similar maximal effects (E_{max} 11.19 ± 0.50) but lower potency (pEC_{50} 8.94 ± 0.01, p < 0.05) than [(*p*F)Phe^4] (Fig. 1). [(*p*F)Phe^4] and N/OFQ-(1–13)NH_2 stimulated GTPγ^{35}S binding was pertussis toxin sensitive (Table 1). The effects of [(*p*F)Phe^4] were competitively antagonised by J-113397 and III-BTD, both of which caused a parallel, concentration-dependent rightward shift in the agonist concentration–response curve (Fig. 2). Schild analysis yielded pA_2 values for J-113397 and III-BTD, as shown in Table 2. Both antagonists were devoid of residual agonist activity.

3.2. Cyclic AMP assays

In whole CHO_{hNOP} cells [(*p*F)Phe^4] produced a concentration-dependent inhibition of forskolin-stimulated cAMP formation with a pEC_{50} and E_{max} of 10.19 ± 0.06 and 102 ± 1%, respectively (Fig. 3). In the same preparation N/

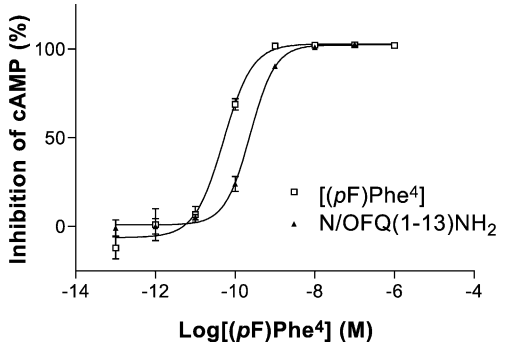

Fig. 3. N/OFQ-(1–13)NH_2 and [(*p*F)Phe^4] inhibit forskolin-stimulated cAMP formation in whole CHO_{hNOP} cells. Data (percentage inhibition) are mean ± S.E.M. (n = 3).

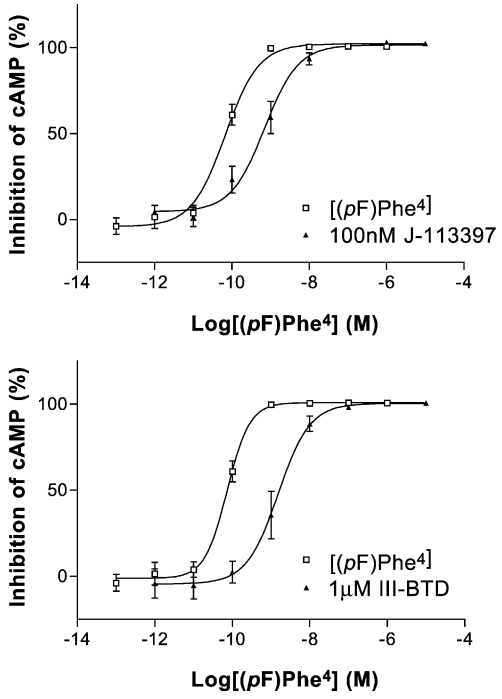

Fig. 4. Competitive antagonism of [(*p*F)Phe^4] mediated inhibition of forskolin stimulated cAMP accumulation by J-113397 (upper panel) and III-BTD (lower panel). Data are mean ± S.E.M. ($n=4$).

OFQ-(1–13)NH_2 also inhibited forskolin-stimulated cAMP formation with a pEC_{50} of 9.6 ± 0.04 (and E_{max} of 103 ± 1%) which was ~ 4 fold less potent ($p<0.05$) than [(*p*F)Phe^4] (Fig. 3). [(*p*F)Phe^4] and N/OFQ-(1–13)NH_2 inhibition of cAMP formation was pertussis toxin sensitive (Table 1). The effects of [(*p*F)Phe^4] were competitively antagonised by J-113397 (100 nM) and III-BTD (1 μM), both of which caused a parallel rightward shift in the agonist concentration–response curve (Fig. 4). pK_B values calculated using the Gaddum–Schild equation are shown in Table 2. Both antagonists were devoid of residual agonist activity.

4. Discussion

In this study, we have clearly demonstrated that [(*p*F)Phe^4] stimulated the binding of GTPγ^{35}S and inhibited forskolin-stimulated cAMP formation in Chinese hamster ovary cells expressing the recombinant human NOP. In both assay systems, [(*p*F)Phe^4] was ~ 4 times more potent than the template peptide N/OFQ-(1–13)NH_2. Moreover, the actions of this novel peptide were pertussis toxin sensitive and competitively inhibited by the NOP-selective antagonist J-113397 and the non-selective antagonist III-BTD.

Studies of the pharmacology of the N/OFQ–NOP system have been greatly hampered by the relative lack of ligands (peptide and non-peptide) with which to make detailed *in vitro* and more importantly *in vivo* studies. For a molecule to be of interest in the therapeutic arena, this needs to be highly selective and preferably non-peptide in nature. Several such molecules have been described and these include the Roche agonist 1*S*,3*aS*)-8-(2,3,3*a*,4,5,6-hexahydro-1*H*-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro [4.5]decan-4-one (Ro64-6198) which displays an impressive anxiolytic profile (Jenck et al., 2000; Dautzenberg et al., 2001) and the antagonist of Banyu, J-113397 (Kawamoto et al., 1999; Ozaki et al., 2000). Several other ligands have been described in the literature with varying degrees of selectivity, e.g., *N*-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl)benzamide hydrochloride (JTC-801, Shinkai et al., 2000; Yamada et al., 2002) and III-BTD (Becker et al., 1999). In general, these molecules have some activity at classical opioid (MOP/DOP/KOP) receptors. For several years, we have been involved in detailed structure–activity relationship studies of the N/OFQ peptide (Guerrini et al., 2000) and have described several modifications of the two pharmacophores in the message domain of the peptide; Phe^1 and Phe^4. For example, [Nphe^1] substitution of the peptide converts N/OFQ from a potent full agonist to a low potency (pA_2 6.0–6.7) antagonist (Calo' et al., 2000b; Hashimoto et al., 2000). In the present study, we have substituted an F-atom into the *para* position of Phe^4 (Guerrini et al., 2001) which has increased the potency of this new peptide with respect to the template.

It is important to confirm that any novel molecule activates the receptor of interest (NOP), utilises the appropriate transduction machinery and displays selectivity. We have competitively reversed the effects of both [(*p*F)Phe^4] and N/OFQ-(1–13)NH_2 with two antagonists; J-113397 and III-BTD and have obtained pA_2/pK_B similar to those obtained in this assay against N/OFQ (Hashiba et al., 2001, 2002) and to those reported in the literature by other groups and in different preparations (Becker et al., 1999; Kawamoto et al., 1999). At this point, it is worthy of further mention that the estimated antagonist potency in GTPγ^{35}S assay is higher than in the cAMP assay. We have no firm explanation for this other than differing buffer/assay systems but should mention that we and others have reported these relative differences previously (Hashiba et al., 2002; Berger et al., 2000). In addition, we have reported that [(*p*F)Phe^4] displays some 3000-fold NOP selectivity over classical opioid receptors, its actions in isolated tissues are unaffected by naloxone (Guerrini et al., 2001; Bigoni et al., 2002) and the cellular actions are completely prevented by pertussis toxin inactivation of $G_{i/o}$. Moreover in a very recent paper we have also shown that the *in vivo* actions of [(*p*F)Phe^4] are longer lasting than the natural ligand N/OFQ (Rizzi et al., 2002a).

As mentioned above there are several antagonist molecules in the literature and recently there has been increased effort directed to the development of agonist molecules for the NOP receptor. This is perhaps best typified by Ro64-6198 (Jenck et al., 2000). [Arg^{14},Lys^{15}]N/OFQ (Okada et al., 2000) was the first peptide analogue of N/OFQ to have greater affinity and potency than N/OFQ itself and has been charac-

terized for its actions *in vitro* at recombinant (Okada et al., 2000) and native (Rizzi et al., 2002b) receptors and *in vivo* in animal studies (Rizzi et al., 2002b). In an attempt to improve agonist potency, Ambo et al. (2001) produced several cyclic N/OFQ analogues. Notably, cyclo[Cys^{10},Cys^{14}]N/OFQ-(1–14)NH_2 was found to display full agonist activity with potency (and binding affinity) comparable to N/OFQ. It was suggested that this template may be useful for further modification. As a consequence of the cyclization process, this product may have improved metabolic stability and *in vivo* studies are eagerly awaited.

In summary, [(*p*F)Phe^4] is a highly potent agonist of NOP receptor that will prove a useful tool in the development of the N/OFQ–NOP receptor system.

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References

Ambo, A., Hamazaki, N., Yamada, Y., Nakata, E., Sasaki, Y., 2001. Structure–activity studies on nociceptin analogues: ORL1 receptor binding and biological activity of cyclic disulfide-containing analogues of nociceptin peptides. J. Med. Chem. 44, 4015–4018.

Becker, J.A.J., Wallace, A., Garzon, A., Ingallinella, P., Bianchi, E., Cortese, R., Simonin, F., Kieffer, B.L., Pessi, A., 1999. Ligands for κ-Opioid and ORL1 receptors identified from a conformationally constrained peptide combinatorial library. J. Biol. Chem. 274, 27513–27522.

Berger, H., Calo', G., Albrecht, E., Guerrini, R., Bienert, M., 2000. [$Nphe^1$]NC(1–13)NH_2 selectively antagonizes nociceptin/orphanin FQ-stimulated G-protein activated in rat brain. J. Pharmacol. Exp. Ther. 294, 428–433.

Bigoni, R., Calo', G., Rizzi, A., Guerrini, R., De Risi, C., Hashimoto, Y., Hashiba, E., Lambert, D.G., Regoli, D., 2000. In vitro characterisation of J-113397, a non-peptide nociceptin/orphanin FQ receptor antagonist. Naunyn-Schmiedeberg's Arch. Pharmacol. 361, 565–568.

Bigoni, R., Rizzi, D., Rizzi, A., Camarda, V., Guerrini, R., Lambert, D.G., Hashiba, E., Berger, H., Salvadori, S., Regoli, D., Calo', G., 2002. Pharmacological characterization of [(pX)Phe^4]nociceptin(1–13)amide analogs: I. In vitro studies. Naunyn-Schmiedeberg's Arch. Pharmacol. DOI: 10.1007/S00210-002-0545-8.

Calo', G., Bigoni, R., Rizzi, A., Guerrini, R., Salvadori, S., Regoli, D., 2000a. Nociceptin/orphanin FQ receptor ligands. Peptides 21, 935–947.

Calo', G., Guerrini, R., Bigoni, R., Rizzi, A., Marzola, G., Okawa, H., Bianchi, C., Lambert, D.G., Salvadori, S., Regoli, D., 2000b. Characterisation of [$Nphe^1$]nociceptin(1–13)NH_2, a new selective nociceptin receptor antagonist. Br. J. Pharmacol. 129, 1183–1193.

Calo', G., Guerrini, R., Rizzi, A., Salvadori, S., Regoli, D., 2000c. Pharmacological characterization of nociceptin and its receptor: a novel therapeutic target. Br. J. Pharmacol. 129, 1261–1283.

Cox, B.M., Chavkin, C., Christie, M.J., Civelli, O., Evans, C., Hamon, M.D., Hoellt, V., Kieffer, B., Kitchen, I., McKnight, A.T., Meunier, J.C., Portoghese, P.S., 2000. Opioid receptors. In: Girdlestone, D. (Ed.), The IUPHAR Compendium of Receptor Characterization and Classification. IUPHAR Media, London, pp. 321–333.

Dautzenberg, F.M., Wichmann, J., Higelin, J., Py-Lang, G., Kratzeisen, C., Malherbe, P., Kilpatrick, G.J., Jenck, F., 2001. Pharmacological characterization of the novel non-peptide orphanin FQ/nociceptin receptor agonist Ro 64-6198: rapid and reversible desensitization of the ORL1 receptor in vitro and lack of tolerance in vivo. J. Pharmacol. Exp. Ther. 298, 812–819.

De Risi, C., Piero Pollini, G., Trapella, C., Peretto, I., Ronzoni, S., Giardina, G.A., 2001. A new synthetic approach to 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-benzimidazol-2-one(J-113397), the first non-peptide ORL-1 receptor antagonist. Bioorg. Med. Chem. 9, 1871–1877.

Giuliani, S., Lecci, A., Maggi, C.A., 2000. Nociceptin and neurotransmitter release in the periphery. Peptides 21, 977–984.

Guerrini, R., Calo', G., Rizzi, A., Bianchi, C., Lazarus, L.H., Salvadori, S., Temussi, P.A., Regoli, D., 1997. Address and message for the nociceptin receptor: a structure–activity study of nociceptin-(1–13)-peptide amide. J. Med. Chem. 40, 1789–1793.

Guerrini, R., Calo', G., Rizzi, A., Bigoni, R., Rizzi, D., Regoli, D., Salvadori, S., 2000. Structure–activity relationships of nociceptin and related peptides: comparison with dynorphin A. Peptides 21, 923–933.

Guerrini, R., Calo', G., Bigoni, R., Rizzi, D., Rizzi, A., Zucchini, M., Varani, K., Hashiba, E., Lambert, D.G., Toth, G., Borea, P.A., Salvadori, S., Regoli, D., 2001. A structure–activity studies of the Phe^4 residue of nociceptin-(1–13)-NH_2: identification of highly potent agonist of the nociceptin/orphanin FQ receptor. J. Med. Chem. 44, 3956–3964.

Hashiba, E., Harrison, C., Calo', G., Guerrini, R., Rowbotham, D.J., Smith, G., Lambert, D.G., 2001. Characterisation and comparison of novel ligands for the nociceptin/orphanin FQ receptor. Naunyn-Schmiedeberg's Arch. Pharmacol. 362, 28–33.

Hashiba, E., Lambert, D.G., Jenck, F., Wichmann, J., Smith, G., 2002. Characterisation of the non-peptide nociceptin receptor agonist, Ro64-6198 in Chinese hamster ovary cells expressing recombinant human nociceptin receptors. Life Sci. 70, 1719–1725.

Hashimoto, Y., Calo', G., Guerrini, R., Smith, G., Lambert, D.G., 2000. Antagonistic effects of [$Nphe^1$]nociceptin(1–13)NH_2 on nociceptin receptor mediated inhibition of cAMP formation in Chinese ovary hamster cells stably expressing the recombinant human nociceptin receptor. Neurosci. Lett. 278, 109–112.

Hawes, B.E., Graziano, M.P., Lambert, D.G., 2000. Cellular actions of nociceptin: transduction mechanisms. Peptides 21, 961–967.

Jenck, F., Wichmann, J., Dautzenberg, F.M., Moreau, J.L., Ouagazzal, A.M., Martin, J.R., Lundstrom, K., Cesura, A.M., Poli, S.M., Roever, S., Kolczewski, S., Adam, G., Kilpatrick, G., 2000. A synthetic agonist at the orphanin FQ/nociceptin receptor ORL1: anxiolytic profile in the rat. Proc. Natl. Acad. Sci. U.S.A. 97, 4938–4943.

Kawamoto, H., Ozaki, S., Itoh, Y., Miyaji, M., Arai, S., Nakashima, H., Kato, T., Ohta, H., Iwasawa, Y., 1999. Discovery of the first potent and selective small molecule opioid receptor-like (ORL1) antagonist: 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3- hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (J-113397). J. Med. Chem. 42, 5061–5063.

Lowry, O.H., Nira, J., Rosebrough, A., Farr, L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J.L., Guillemot, J.C., Ferrara, P., Monserrat, B., Mazarguil, H., Vassart, G., Parmentier, M., Costentin, J., 1995. Isolation and structure of the endogenous agonist of opioid receptor-like ORL_1 receptor. Nature 377, 532–535.

Mogil, J.S., Pasternak, G.W., 2001. The molecular and behavioural pharmacology of the orphanin FQ/nociceptin peptide and receptor family. Pharmacol. Rev. 53, 381–415.

Okada, K., Sujaku, T., Chuman, Y., Nakashima, R., Nose, T., Costa, T., Yamada, Y., Yokoyama, M., Nagahisa, A., Shimohigashi, Y., 2000. Highly potent nociceptin analog containing the Arg–Lsy triple repeat. Biochem. Biophys. Res. Commun. 278, 493–498.

Okawa, H., Nicol, B., Bigoni, R., Hirst, R.A., Calo, G., Guerrini, R., Rowbotham, D.J., Smart, D., McKnight, A.T., Lambert, D.G., 1999. Comparison of the effects of [Phe$^1\Psi$(CH_2-NH)Gly^2]Nociceptin(1–13)NH_2 in rat brain, rat vas deferens and CHO cells expressing recombinant human nociceptin receptors. Br. J. Pharmacol. 127, 123–130.

Ozaki, S., Kawamoto, H., Itoh, Y., Miyaji, M., Iwasawa, Y., Ohta, H., 2000. A potent and highly selective nonpeptidyl nociceptin/orphanin FQ receptor (ORL1) antagonist: J-113397. Eur. J. Pharmacol. 387, R17–R18.

Reinscheid, R.K., Nothacker, H.-P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma Jr., F.J., Civelli, O. 1995. Orphanin FQ: a neuropeptide that activates an opioid like G protein-coupled receptor. Science 270, 792–794.

Rizzi, A., Bonaria Salis, M., Ciccocioppo, R., Marzola, G., Bigoni, R., Guerrini, R., Massi, M., Madeddu, P., Salvadori, S., Regoli, D., Calo', G., 2002a. Pharmacological characterization of [(pX)Phe^4]nociceptin(1–13)amide analogs: II. In vivo studies. Naunyn-Schmiedeberg's Arch. Pharmacol. DOI: 10.1007/S00210-002-0548-7.

Rizzi, D., Rizzi, A., Bigoni, R., Camarda, V., Marzola, G., Guerrini, R., De Risi, C., Regoli, D., Calo', G., 2002b. [Arg(14),Lys(15)]nociceptin, a highly potent agonist of the nociceptin/orphanin FQ receptor: in vitro and in vivo studies. J. Pharmacol. Exp. Ther. 300, 57–63.

Schlicker, E., Morari, M., 2000. Nociceptin/orphanin FQ and neurotransmitter release in the central nervous system. Peptides 21, 1023–1029.

Shinkai, H., Ito, T., Iida, T., Kitao, Y., Yamada, H., Uchida, I., 2000. 4-Aminoquinolines: novel nociceptin antagonists with analgesic activity. J. Med. Chem. 43, 4667–4677.

Yamada, H., Nakamoto, H., Suzuki, Y., Ito, T., Aisaka, K., 2002. Pharmacological profiles of a novel opioid receptor-like1 (ORL_1) receptor antagonist, JTC-801. Br. J. Pharmacol. 135, 323–332.