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Recognition and activation of the opioid receptor-like ORL1 receptor by nociceptin, nociceptin analogs and opioids

Jean-Luc Butour, Christiane Moisand, Honoré Mazarguil, Catherine Mollereau, Jean-Claude Meunier *

Unité de Neuropharmacologie Moléculaire, Institut de Pharmacologie et de Biologie Structurale, Centre National de la Recherche Scientifique, UPR 9062, 205 route de Narbonne, 31077 Toulouse Cedex, France

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Abstract

Nociceptin, also known as orphanin FQ, was recently identified as the naturally occurring agonist of orphan opioid receptor-like ORL1 receptor (Meunier et al., 1995, Nature 377, 532; Reinscheid et al., 1995, Science 270, 792). Nociceptin is a heptadecapeptide which, although it resembles dynorphin A, the endogenous agonist of the κ-opioid receptor, displays very low potency in competing with binding of [^{3}H]diprenorphine to or inhibiting adenylate cyclase via μ-, δ- and κ-opioid receptors. Tritium-labeled nociceptin ([^{3}H]nociceptin) was used here to establish a pharmacological profile in vitro of the ORL1 receptor. In membranes from recombinant Chinese hamster ovary (CHO) cells expressing the ORL1 receptor, equilibrium binding of [^{3}H]nociceptin is highly specific, saturable (B_{max} in the range 1.3–1.8 pmol/mg protein) and of high affinity ($K_d \approx 0.1$ nM). It is selectively decreased in the presence of Na^+ ions and/or of the GTP analog 5′-guanylylimido-diphosphate, an allosteric regulation that is analogous to that of opiate binding to opioid receptors. A few opiates, namely lofentanil, a 4-anilinopiperidine derivative and etorphine, a 6,14-*endo*-ethenotetrahydrothebaine derivative, were found to be quite potent not only in competing with binding of [^{3}H]nociceptin at the ORL1 receptor but also in inhibiting forskolin-induced accumulation of cyclic AMP in intact recombinant CHO cells. In a preliminary attempt to delineate active parts of the neuropeptide, nociceptin analogs were also tested, including N- and C-terminal truncation products. Our results suggest that the highly basic, internal core of nociceptin might be essential in conferring on the peptide both affinity for and activity at the ORL1 receptor. In this respect, the message and address division of dynorphin A, nociceptin's closest structural analog, do not seem to apply to nociceptin.

Keywords: [^{3}H]Nociceptin; Nociceptin receptor; Radioreceptor assay; Adenylylcyclase, inhibition; Drug screening

1. Introduction

Nociceptin (Meunier et al., 1995) or orphanin FQ (Reinscheid et al., 1995) is the endogenous agonist of opioid receptor-like ORL1 receptor, an orphan G protein-coupled receptor whose human (Mollereau et al., 1994) and murine (Fukuda et al., 1994; Chen et al., 1994; Bunzow et al., 1994; Wang et al., 1994; Wick et al., 1994; Nishi et al., 1994; Lachowicz et al., 1995) complementary DNAs had been previously cloned. The ORL1 receptor displays substantial sequence homologies with opioid receptors, especially in the putative membrane-spanning domains and intracellular loops. In Chinese hamster ovary (CHO) cells stably expressing ORL1, the receptor mediates inhibition of forskolin-induced accumulation of cyclic AMP by etorphine, but at concentrations of the opiate considerably higher than those required to activate opioid receptors (Mollereau et al., 1994). In situ hybridization (Mollereau et al., 1994; Bunzow et al., 1994; Chen et al., 1994; Fukuda et al., 1994) and immuno-histochemistry (Anton et al., 1996) have located ORL1 in many areas of the central nervous system, including limbic areas, hypothalamus, pons and spinal cord, indicating that the receptor may regulate a number of central processes, including learning and memory, attention and emotions, homeostasis and sensory perception. Indeed, repeated intracerebroventricular (i.c.v.) treatment with anti-sense oligonucleotide to ORL1 messenger RNA induces analgesia in mice (Meunier et al., 1995).

The natural ligand of the orphan receptor was identified as a component of brain extracts that inhibits adenylyl cyclase in recombinant CHO cells expressing ORL1. It is a

* Corresponding author. Tel.: (33-5) 6117-5981; Fax: (33-5) 6117-5994; e-mail: jcm@ipbs.fr

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heptadecapeptide whose sequence (FGGFTGARKSARKLANQ) is related, however distantly, to that of dynorphin A, the putative endogenous agonist of the κ-opioid receptor. The peptide displays nanomolar potency in the ORL1-mediated adenylyl cyclase inhibition assay and, when i.c.v.- or intrathecally-administered in mice, induces hyperalgesia (Meunier et al., 1995; Reinscheid et al., 1995) and stimulates locomotion and curiosity (Florin et al., 1996). Interestingly, nociceptin was recently reported to exert pronociceptive effects too, upon systemic injection in the land snail, *Cepeae nemoralis* (Kavaliers and Perrot-Sinal, 1996).

As a first step toward the design of better ORL1 receptor ligands, we have examined here the binding properties of the recombinant receptor stably expressed in CHO cells. Two screening tests were used: the ability of unlabeled effectors (i) to compete with equilibrium binding of [^{3}H]nociceptin in CHO(ORL1) cell-derived membranes and (ii) to elicit ORL1-mediated inhibition of forskolin-induced accumulation of cAMP in intact CHO(ORL1) cells. We find in particular that (i) the opiate lofentanil, a 4-anilinopiperidine derivative, is a quite potent ORL1 receptor agonist and (ii) the highly basic, internal core of nociceptin might be essential in conferring on the peptide both affinity and agonist activity at the ORL1 receptor, thus invalidating the notion that the message and address division of dynorphin A, nociceptin's closest structural analog, applies to nociceptin.

2. Materials and methods

2.1. Materials

[^{3}H]Nociceptin (0.85 TBq/mmol; 23 Ci/mmol) was produced at Amersham (Amersham, UK) by catalytic dehalogenation of [pBr-Phe^1,pBr-Phe^4]nociceptin in the presence of tritium gas. The tritium-labeled peptide had the expected mass, as assessed by mass spectrometry, and was undistinguishable from the unlabeled peptide, as assessed by high-pressure liquid chromatography. Dynorphin A and all nociceptin-derived peptides were synthesized in the laboratory, in solid phase, except des-Phe^1-nociceptin, a generous gift from Dr M.-C. Fournier-Zaluski (Paris). [Leu^5]enkephalin, [Met^5]enkephalin, α-neoendorphin, β-endorphin, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin (DAMGO) and [D-Thr^2,Leu^5,Thr^6]enkephalin (DTLET) were purchased from Bachem (Basel, Switzerland). Morphine and naloxone were from Francopia (Paris, France), etorphine and diprenorphine from Reckitt and Colman (Kingston upon Hull, UK), ethylketocyclazocine from Sterling Winthrop (Rensselaer, NY, USA), *nor*-binaltorphimine and *trans*-(1*S*,2*S*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride (U-50488) from Research Biochemicals (Natick, MA, USA). Lofentanil and fentanyl were kindly provided by Dr. J. Leysen (Janssen Pharmaceuticals, Beerse, Belgium). 5′-Guanylylimidodiphosphate (GppNHp), polyethyleneimine, bovine serum albumin (fraction V, protease-free) and alumina (activity grade 1, type WA-1: acid) were from Sigma (St Louis, MO, USA).

2.2. Binding and competition studies

CHO cells stably expressing the ORL1 receptor were grown and a membrane fraction thereof was prepared as previously described (Mollereau et al., 1996). To minimize loss of material, presumably due to nonspecific adsorption, the following precautions were taken: (i) stock solutions as well as intermediate dilutions of peptides were in 0.1 mg/ml protease-free bovine serum albumin, in polypropylene tubes, (ii) binding and competition studies were carried out in polypropylene tubes in the presence of 0.1 mg/ml protease-free bovine serum albumin and (iii) glass fiber disks (Whatman GF/B) that had been soaked in polyethyleneimine (0.33%, v/v) were used.

For equilibrium binding studies, each incubation mixture (0.5 ml, in triplicate) consisted of 30–50 μg membrane protein and [^{3}H]nociceptin at the desired concentration (in the range 0.05–2 nM) in 50 mM Tris-HCl, pH 7.5. Nonspecific binding was determined in the presence of 1 μM unlabeled nociceptin. Following a 1 h incubation at 25°C, unbound radioligand was removed by rapid suction through glass fiber filters (see above) and rinsing 3 times with 4 ml 10 mM Tris-HCl buffer (pH 7.5). Filter-bound radioactivity was counted in 3 ml of Beckman Ready Protein$^+$ cocktail using a Kontron MR 300 liquid scintillation counter.

For competition studies, each incubation mixture (0.5 ml, in triplicate) consisted of 30–50 μg membrane protein, [^{3}H]nociceptin at the fixed concentration of 1 nM, and the unlabeled ligand at the desired concentration, in 50 mM Tris-HCl, pH 7.5. Total (no unlabeled ligand) and nonspecific (+1 μM unlabeled nociceptin) bindings were determined in sextuplicate. Bound radioactivity was isolated and measured as described above.

2.3. Assay for intracellular cAMP

Sterile hemolysis tubes were seeded with about 200 000 recombinant CHO cells in culture medium and incubated overnight at 37°C. The culture medium was then removed and replaced by 200 μl of HEPES-buffered Krebs-Ringer saline (KRH: 124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM HEPES, 8 mM glucose, 0.5 mg/ml bovine serum albumin; pH 7.4) containing 0.1 μM adenine and 0.6 μCi [^{3}H]adenine (24 Ci/mmol, Amersham). After 1 h at 37°C, the cells were rinsed with 400 μl of KRH and 180 μl of fresh KRH was added to each tube. Intracellular accumulation of cAMP was initiated by the addition of 20 μl of KRH containing 100 μM forskolin (Sigma), 1 mM 3-isobutyl-1-methylxanthine (Sigma), 1 mM 4-(3-butoxy-4-methoxybenzyl)-

2-imidazolidinone (Ro20-1724, Biomol. Res.), and the ligand(s) to be tested at 10-fold the desired final concentration. After exactly 10 min at 37°C, the reaction was stopped by the addition of 20 μl 2.2 M HCl and rapid mixing (Vortex), and the [^{3}H]cAMP content of each tube was determined by selective batch elution on columns of 0.65 g acidic alumina, essentially as described by Alvarez and Daniels (1992).

2.4. Analysis of the data

Nonlinear regression analyses of the data were performed using InPlot ver 4.03 from Graphpad Software (San Diego, CA, USA).

3. Results

3.1. Equilibrium saturation binding of [^{3}H]nociceptin in membrane preparations from recombinant CHO(ORL1) cells

In membrane preparations from recombinant CHO(ORL1) cells, equilibrium binding of [^{3}H]nociceptin was specific, dose-dependent and saturable (Fig. 1). The Scatchard transformation of the data was linear, indicating a homogenous population of binding sites (B_{max} in the range 1.3–1.8 pmol/mg protein, depending on membrane preparation) with high affinity for [^{3}H]nociceptin: K_d = 0.11 ± 0.03 nM (n = 5). Nonspecific binding was low, and amounted to about 15% of total binding at a nearly saturating concentration (1 nM) of radioligand. Nociceptin's K_d value could not be calculated from rate constants since association of [^{3}H]nociceptin was too rapid to be followed using the so-called 'rapid' filtration assay (data not shown).

Binding of [^{3}H]nociceptin was found to be sensitive to Na^+ and GppNHp, a metabolically stable analogue of GTP (Fig. 2). In the presence of 120 mM NaCl, equilibrium saturation binding of [^{3}H]nociceptin became clearly biphasic, about one third of the sites remaining in a high-affinity state, the other two thirds being converted into a 20- to -30-fold lower affinity state. This effect appeared to be specific since equilibrium binding of [^{3}H]nociceptin was unaffected in the presence of 120 mM choline chloride (not shown). GppNHp (100 μM) alone significantly affected neither the affinity nor the binding capacity of [^{3}H]nociceptin, yet, when added together with NaCl, further reduced the affinity of those high-affinity sites which remained in the presence of Na^+ alone. Such allosteric regulation was highly reminiscent of that observed for binding of opiates at opioid receptors (see, for example, Francés et al., 1985).

3.2. Ability of various unlabelled drugs and nociceptin analogs to compete with binding of [^{3}H]nociceptin in membrane preparations from CHO(ORL1) cells

A representative set of opioid ligands was tested for potency to inhibit binding of [^{3}H]nociceptin at ORL1 sites in membrane preparations from recombinant CHO(ORL1) cells. This included (i) the non- or poorly type-selective, nonpeptide agonists morphine and etorphine (4,5-epoxymorphinans), ethylketocyclazocine (a benzomorphan), fentanyl and lofentanil (4-anilinopiperidines), and the antagonists naloxone and diprenorphine (4,5-epoxymorphinans), (ii) the κ-selective agonist and antagonist U-50488 (an arylacetamide) and *nor*-binaltorphimine, respectively, and (iii) the natural opioid peptides [Leu^5]- and [Met^5]enkephalin, α-neoendorphin, dynorphin A and β-endorphin, and the μ- and δ-selective agonists, DAMGO and DTLET, respectively. Fig. 3 and Table 1 show that only three of these opioid ligands, namely lofentanil, dynorphin A and etorphine, displayed substantial apparent affinity (K_i < 1 μM) for the ORL1 binding site. Lofentanil was the most potent (K_i = 24 nM) while its close structural analog, fentanyl, was inactive (see Section 4).

In a preliminary attempt to delineate the structure–activity relationships of nociceptin, we next examined the potency of various analogs of nociceptin to compete with equilibrium binding of [^{3}H]nociceptin at the ORL1 recep-

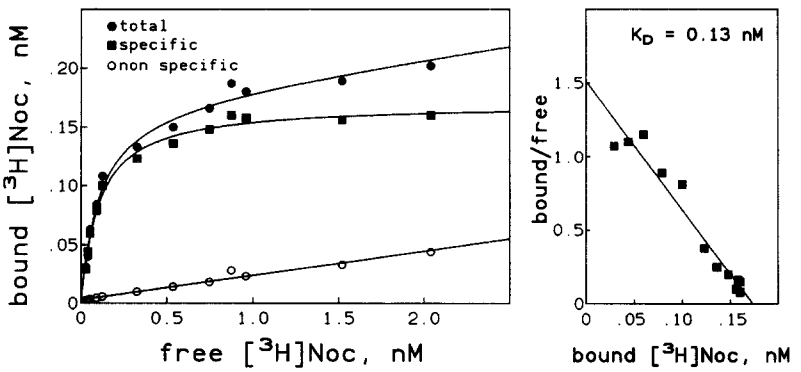

Fig. 1. Equilibrium binding of [^{3}H]nociceptin (Noc) in a membrane fraction from recombinant CHO cells expressing the ORL1 receptor. Binding was at 25°C in 50 mM Tris-HCl, pH 7.4. Left: Representative saturation isotherm. Nonspecific binding was measured in the presence of 1 μM unlabeled nociceptin. Right: Scatchard transformation of the data. 'Bound' refers to specifically bound [^{3}H]nociceptin.

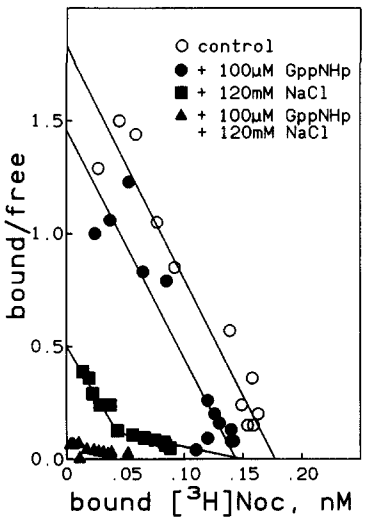

Fig. 2. Effects of Na^+ and GppNHp on equilibrium saturation binding of [^{3}H]nociceptin (Noc) in a membrane fraction from recombinant CHO cells expressing the ORL1 receptor. Scatchard transformation of the data. 'Bound' refers to specifically bound [^{3}H]nociceptin. Nonspecific binding was measured in the presence of 1 μM unlabeled nociceptin.

tor site (Fig. 4 and Table 1). Replacing the N-terminal Phe residue in nociceptin by Tyr resulted in a peptide, [Tyr^1]nociceptin, that was nearly as potent as the parent peptide. On the contrary, removing the N-terminal Phe residue to generate des-Phe^1-nociceptin decreased dramatically (≥ 2000-fold) the peptide's apparent affinity for ORL1. Removal of C-terminal amino acids also resulted in a substantial loss of affinity for ORL1: nociceptin-(1–13)

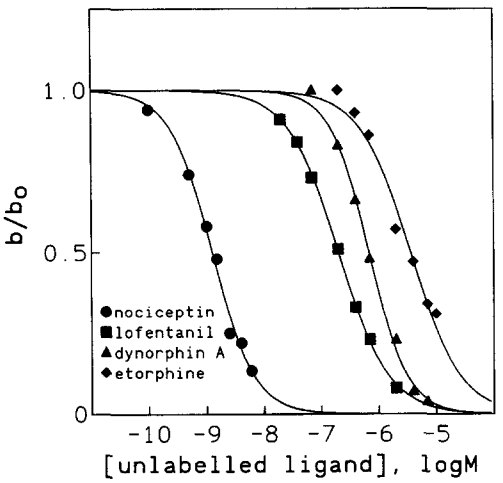

Fig. 3. Inhibition by lofentanil, dynorphin A and etorphine of specific binding of [^{3}H]nociceptin in a membrane fraction from recombinant CHO cells expressing the ORL1 receptor. Nociceptin's inhibition curve is also shown as the reference. [^{3}H]Nociceptin was used at the fixed concentration of 1 nM. 'b_0' and 'b' refer to specifically bound radioligand in the absence and presence of inhibitor, respectively. Apparent Hill coefficients were all in the range of 0.9–1.1, indicating a homogenous population of binding sites. The following opiates, opioid peptides and antagonists were found to be inactive (K_i > 10 μM) in this assay: morphine, fentanyl, ethylketocyclazocine, naloxone, diprenorphine, *nor*-binaltorphimine, DAMGO, DTLET, U-50488, [Met^5]enkephalin, [Leu^5]enkephalin, α-neoendorphin and β-endorphin.

Table 1
Parameters of recognition (K_i) and activation (ED_{50}) of the opioid receptor-like ORL1 receptor by nociceptin (Noc), nociceptin analogs and opioids

Effector	K_i (nM)	ED_{50} (nM)
FGGFTGARKSARKLANQ (Noc)	0.13	0.8
YGGFTGARKSARKLANQ	0.26	1.1
FGGFTGARKSARK	4.0	7.8
FGGFTGARKSAR	145	67
FGGFTGARKSA	1300	3400
FGGFTGARKS	2150	3900
FGGFTGARK	4300	> 10000
FGGFTGAR	9700	> 10000
FGGFTGA	> 10000	≫ 10000
FGGFTG	> 10000	≫ 10000
FGGFT	> 10000	≫ 10000
FGGF	> 10000	≫ 10000
GGFTGARKSARKLANQ	275	1400
GARKSARKLANQ	20	39
RKLANQ	49	790
Lofentanil	24	7.2
Fentanyl	> 1000	> 10000
Dynorphin A	110	> 10000
Etorphine	530	460

Each K_i value was calculated from the concentration (IC_{50}) of unlabeled effector causing 50% inhibition of binding of [^{3}H]nociceptin in CHO(ORL1) cell membranes (see Figs. 3 and 4), using the equation of Cheng and Prussof (1973). ED_{50} is the concentration of unlabeled effector causing half-maximal inhibition of forskolin-induced accumulation of cAMP in intact CHO(ORL1) cells (see Figs. 5 and 6). Each value is the mean of two or three values obtained from two or three independent experiments and differing by ≤ 20%.

exhibited only 1/30th the potency of the parent peptide, and further stepwise removal of individual amino acids down to nociceptin-(1–6) further decreased the apparent affinity. There were no evident discontinuities in the affinity vs. peptide length relationship (not shown), making it

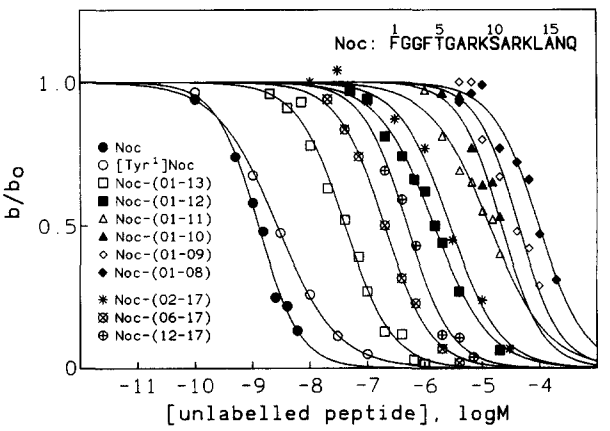

Fig. 4. Inhibition by nociceptin analogs of specific binding of [^{3}H]nociceptin in a membrane fraction from recombinant CHO cells expressing the ORL1 receptor. Nociceptin's inhibition curve is also shown as the reference. [^{3}H]Nociceptin was used at the fixed concentration of 1 nM. 'b_0' and 'b' refer to specifically bound radioligand in the absence and presence of inhibitor, respectively. Apparent Hill coefficients were all in the range of 0.9–1.1, indicating a homogenous population of binding sites.

difficult to identify particularly important residues. Surprisingly enough, the C-terminal nociceptin fragments –(12–17) and –(6–17) retained fairly high affinity for ORL1 with apparent dissociation constants in the range of 20–50 nM.

3.3. Ability of various unlabeled drugs and nociceptin derivates to inhibit forskolin-induced accumulation of cAMP in intact CHO(ORL1) cells

Since binding alone cannot discriminate agonists and antagonists, we also tested the unlabeled ligands for their ability to inhibit forskolin-induced accumulation of cAMP in intact CHO(ORL1) cells. Fig. 5 and Table 1 show that opioid ligands that did not bind ORL1 with measurable apparent affinity (K_i < 1 μM) were also inefficient in the functional assay. In contrast, lofentanil and etorphine maximally inhibited the production of cAMP, thus acting as full agonists of the ORL1 receptor. Curiously, dynorphin A, although it displayed higher apparent affinity than etorphine for ORL1, was somewhat less efficient than the opiate in inhibiting cAMP synthesis, an indication that the peptide could be endowed with ORL1 receptor antagonist properties.

Concerning nociceptin analogs (Fig. 6 and Table 1), [Tyr^1]nociceptin was as active as nociceptin, causing half-maximal inhibition of cAMP synthesis at about 1 nM in the recombinant cell line. des-Phe^1-nociceptin was inactive (ED_{50} > 1 μM). Stepwise C-terminal amino acid deletion of nociceptin, from nociceptin-(1–13) down to nociceptin-(1–4) resulted in peptides displaying full ORL1 agonist activity but with progressively increasing ED_{50}s. C-terminally truncated nociceptins ranked in the same order of potency in inhibiting binding of [^{3}H]nociceptin in CHO(ORL1) cell membranes and cAMP synthesis in intact CHO(ORL1) cells. Here, removal of Arg^{12} and/or Lys^{13} appeared to be particularly deleterious to nociceptin's

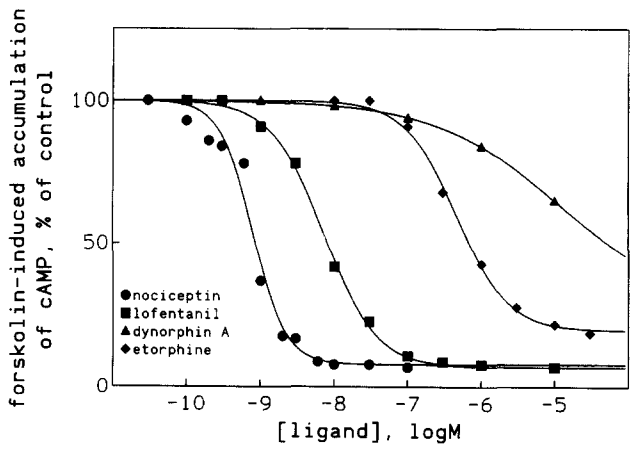

Fig. 5. Inhibition by lofentanil, dynorphin A and etorphine of forskolin-induced accumulation of cAMP in intact recombinant CHO cells expressing the ORL1 receptor. Nociceptin's inhibition curve is also shown as the reference. Assay for cAMP in intact cells was as described in Section 2.

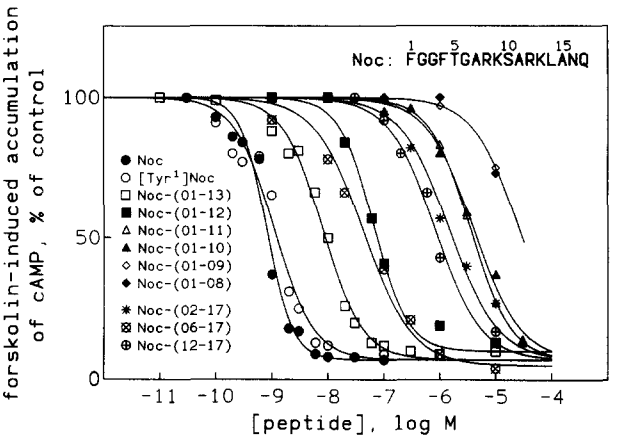

Fig. 6. Inhibition by nociceptin analogs of forskolin-induced accumulation of cAMP in intact recombinant CHO cells expressing the ORL1 receptor. Nociceptin's inhibition curve is also shown as the reference. Assay for cAMP in intact cells was as described in Section 2.

activity. The C-terminal fragments nociceptin-(6–17) and nociceptin-(12–17), especially the former, behaved as quite potent ORL1 receptor agonists, in keeping with their relatively high apparent affinity for the receptor (see above).

4. Discussion

In membranes from recombinant CHO cells expressing high levels of ORL1 and provided that certain precautions are taken (see Section 2.2), [^{3}H]Nociceptin displays specific, high-affinity (K_d = 0.1 nM) binding. Therefore, the recombinant CHO cell line and [^{3}H]nociceptin can be used in a specific radioreceptor assay for the screening of chemical files, in search of potent, nonpeptide agonists and antagonists of the ORL1 receptor. Recently, Dooley and Houghten (1996) have reported somewhat different binding characteristics of [^{3}H]nociceptin, however in rat brain membranes: a much slower rate of association and a 50-fold higher (5 vs. 0.1 nM) equilibrium dissociation constant. These differences may be accounted for by the use of different binding assay conditions as well as of different sources of ORL1 sites (nerve tissue vs. recombinant non-neural cells). In the latter respect, however, it may be remembered that opioid receptors do not show significantly different binding properties in nerve tissue and recombinant CHO cell membranes (Raynor et al., 1994).

Also reported here is the allosteric inhibition of nociceptin binding by Na^+ and GppNHp. Since no ORL1 receptor antagonist is available at present, it is not known whether such regulation selectively affects binding of agonists, as has long been known to be the case for opioid receptors (Pert et al., 1973). However, selective sensitivity to sodium ions of agonist binding at opioid (Kong et al., 1993a) and other (Horstman et al., 1990; Kong et al.,

1993b) receptors appears to require a conserved amino acid residue which is also present in the ORL1 receptor: Asp in the putative second membrane-spanning domain. It may therefore be anticipated that the ORL1 receptor is amenable to the same allosteric regulation as are opioid receptors.

In intact recombinant CHO(ORL1) cells, the ORL1 receptor was initially demonstrated to mediate inhibition of adenylyl cyclase by the opiate, etorphine (Mollereau et al., 1994). Although the doses of opiate which maximally inhibited the cyclase via ORL1 were substantially higher than those required to inhibit the enzyme via opioid receptors, this indicated that the ORL1 receptor did recognize and respond to opiates: i.e., that the ORL1 receptor contains a functional binding pocket whose structure resembles the opioid binding pocket of opioid receptors. This notion was supported here by the fact that not only etorphine, but also dynorphin A and lofentanil, bound ORL1, but with much lower affinity (2 to 3 orders of magnitude) than they do opioid receptors. The ability of dynorphin A to interact with ORL1 could be accounted for by the presence of a highly acidic second exofacial loop in the receptor's putative secondary structure. Interestingly, lofentanil's affinity for ORL1 was quite high (K_i in the range 20–30 nM) while that of fentanyl, a close structural analog of lofentanil, was very low ($K_i > 1$ μM), indicating that the ORL1 receptor differentiates well different substituents on the 4-anilinopiperidine motif common to the two drugs. Identifying these substituents may therefore provide a first clue towards the design of nonpeptide ORL1 ligands, based on the 4-anilinopiperidine core.

As for nociceptin analogs, a few were tested here to delineate simpler, active peptides, and determine to what extent the 'message' and 'address' divisions of dynorphin A (Chavkin and Goldstein, 1981) apply to nociceptin, as initially proposed by Meunier et al. (1995). An aromatic ring at position 1 appeared to be important for nociceptin recognition by ORL1 since replacing the N-terminal Phe residue by Tyr was without effect while removing this residue resulted in a dramatically reduced affinity. C-terminal truncation, too, appeared to be deleterious to both affinity and activity of nociceptin: deletion of the C-terminal tetrapeptide LANQ, and moreover further removal of Lys^{13}, generated peptides with markedly reduced potency at the ORL1 receptor. These results are to be compared with the quite extensive ones that have recently been published by Dooley and Houghten (1996) and Reinscheid et al. (1996). Although the two groups have used similar approaches to the structure–activity relationship of orphanin FQ (nociceptin), including Ala scanning and more or less systematic truncations from both ends of the peptide, they reach opposite conclusions. Dooley and Houghten (1996) claim that the nociceptin sequence may be subdivided into the same 'message' and 'address' moieties as dynorphin A, the N-terminal FGGF sequence representing the message, and the highly basic C-terminal portion the address. In contrast, Reinscheid et al. (1996) conclude that the ORL1 receptor recognizes different parts of the nociceptin molecule and requires the complete peptide structure for biological activity. However, a major difference between the two sets of data is that Dooley and Houghten (1996) have tested C-terminally amidated nociceptin fragments while Reinscheid et al. (and ourselves) have not. Along this line, it was previously shown by Chavkin and Goldstein (1981) that the mere introduction of a negatively charged acidic group into a novel position as it occurs in non-C-terminally esterified, truncated dynorphin-(1–13) fragments adversely affected potency. Thus, stepwise shortening dynorphin-(1–13) down to dynorphin-(1–7) resulted in a steeper decrease in potency (8- vs. 71-fold) for esterified than for nonesterified peptides, in the guinea pig ileum myenteric plexus–longitudinal muscle bioassay. It is therefore possible, by analogy, that omitting to neutralize the C-terminal negative charge of C-terminally truncated nociceptin fragments may have amplified the loss of affinity in comparison with amidated peptides. However, nonesterified dynorphin A and its C-terminally deleted fragments down to as short as dynorphin-(1–7) exhibit equally high affinity for opioid receptors (Mansour et al., 1995). In other words, deletion of as many as the ten C-terminal amino acids from dynorphin A does not affect the peptide's affinity, and this in spite of repositioning of a negative charge. For comparison, we find that removing just the tetrapeptide Leu–Ala–Asn–Gln-OH from the nociceptin sequence results in a tridecapeptide with 1/30th the affinity of the parent peptide for the ORL1 receptor, and that nociceptin-(1–7) is totally inactive, indicating that nociceptin does not bind ORL1 in the same way as dynorphin A does opioid receptors. In fact, the interesting notion here emerges from the observation that the active nociceptin fragments, although less potent than the parent peptide, always contain the positively charged sequence of the molecule. For instance, not only nociceptin-(1–13) but also, and quite unexpectedly, nociceptin-(6–17) and -(12–17), exhibit fairly high apparent affinity and full agonist activity at the ORL1 receptor. Curiously, however, Reinscheid et al. (1996) have reported that nociceptin-(10–17) neither bound nor activated the ORL1 receptor. This apparent discrepancy is difficult to explain in terms of the different primary structures of the N-terminally truncated analogs, and might be better explained on the basis of different (i.e., active vs. inactive) conformations.

In conclusion, the present study has shown that, in spite of the dual structural homology of the ORL1 and opioid receptors on the one hand and their endogenous ligands on the other, nociceptin does not bind ORL1 in the same way as dynorphin A does opioid receptors. In particular, the significant part of nociceptin in terms of both receptor recognition and activation may reside in its basic core. Obviously, a more systematic study of the structure–activity relationships of nociceptin is needed in order to validate this hypothesis.

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