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Characterization of [Nphe^1]nociceptin(1-13)NH_2, a new selective nociceptin receptor antagonist

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1 Nociceptin (orphanin FQ) is a novel neuropeptide capable of inducing a variety of biological actions *via* activation of a specific G-protein coupled receptor. However, the lack of a selective nociceptin receptor antagonist has hampered our understanding of nociceptin actions and the role of this peptide in pathophysiological states. As part of a broader programme of research, geared to the identification and characterization of nociceptin receptor ligands, we report that the novel peptide [Nphe^1]nociceptin(1-13)NH_2 acts as the first truly selective and competitive nociceptin receptor antagonist and is devoid of any residual agonist activity.

2 [Nphe^1]nociceptin(1-13)NH_2 binds selectively to recombinant nociceptin receptors expressed in Chinese hamster ovary (CHO) cells (pK_i 8.4) and competitively antagonizes the inhibitory effects of nociceptin (i) on cyclic AMP accumulation in CHO cells (pA_2 6.0) and (ii) on electrically evoked contractions in isolated tissues of the mouse, rat and guinea-pig with pA_2 values ranging from 6.0 to 6.4.

3 [Nphe^1]nociceptin(1-13)NH_2 is also active *in vivo*, where it prevents the pronociceptive and antimorphine actions of intracerebroventricularly applied nociceptin, measured in the mouse tail withdrawal assay. Moreover, [Nphe^1]nociceptin(1-13)NH_2 produces *per se* a dose dependent, naloxone resistant antinociceptive action and, at relatively low doses, potentiates morphine-induced analgesia.

4 Collectively our data indicate that [Nphe^1]nociceptin(1-13)NH_2, acting as a nociceptin receptor antagonist, may be the prototype of a new class of analgesics.
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Keywords: Nociceptin; [Nphe^1]nociceptin(1-13)NH_2; nociceptin receptor antagonist; native and recombinant receptors; isolated tissues; mouse tail withdrawal assay; morphine, analgesia

Abbreviations: CHO, Chinese hamster ovary; DPDPE, [D-Pen^{2,5}]enkephalin; [F/G]NC(1-13)NH_2, [Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin(1-13)NH_2; NalBzoH, naloxone benzoylhydrazone; [Nphe^1]NC(1-13)NH_2, [Nphe^1]nociceptin(1-13)NH_2

Introduction

Nociceptin or orphanin FQ is a newly discovered neuropeptide (Meunier *et al*., 1995; Reinscheid *et al*., 1995) which shows high structural homology with opioid peptides, especially dynorphin A. Nociceptin activates a specific receptor which has been cloned in man and animals and has been shown to be structurally similar to opioid receptors (see for a review Meunier, 1997). At the cellular level, the nociceptin receptor has been shown to act through the same intracellular mechanisms as classical opioid receptors, namely the inhibition of adenylyl cyclase (Meunier *et al*., 1995; Reinscheid *et al*., 1995), the activation of potassium channels (Connor *et al*., 1996a) and/or inhibition of calcium channels (Connor *et al*., 1996b). The nociceptin receptor has been referred to (by different groups of investigators) as ORL1, LC132, ROR (see for a review Meunier, 1997). Based on the structural and transductional similarities between receptors for nociceptin and those for opioids, at the recent International Congress of Pharmacology in Munich, M. Hamon (Hamon, 1998) proposed to include the nociceptin receptor in the opioid receptor family with the name of OP_4. Since we agree with this proposal, throughout this study we will refer to the nociceptin receptor as OP_4.

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In vitro and *in vivo* studies have demonstrated that nociceptin mediates a variety of biological actions (see for reviews Civelli *et al*., 1998; Meunier, 1997). A summary of findings reported to date indicate that: nociceptin induces analgesia when administered intrathecally, while it causes hyperalgesia and reversal of opioid induced analgesia when given intracerebroventricularly; nociceptin stimulates food intake and produces anxiolysis. Depending on the dose, nociceptin stimulates or inhibits locomotor activity. Nociceptin inhibits long term potentiation, memory processes, induces bradycardia, hypotension, and diuresis. In addition, nociceptin inhibits neurotransmitter release both at central and peripheral sites.

The involvement of OP_4 receptor in some biological actions mediated by nociceptin has been demonstrated (i) with antisense oligonucleotides directed against the receptor, measuring as parameters the nociceptin-induced stimulation of food intake (Leventhal *et al*., 1998) or the inhibition of analgesia induced by electroacupuncture (Tian *et al*., 1997) or by morphine (Zhu *et al*., 1997); (ii) by performing experiments in OP_4 receptor knockout mice and measuring nociceptin evoked hyperalgesia and inhibition of locomotor activity (Nishi *et al*., 1997; Noda *et al*., 1998), or nociceptin induced inhibition of long term potentiation and memory processes (Manabe *et al*., 1998). To date, few molecules have been

reported to act as OP_4 antagonists: thus, Kobayashi *et al*. (1997) showed that some sigma ligands, such as carbetapentane and rimcazole, act as antagonists on the recombinant OP_4 receptor by blocking, in a concentration-dependent manner, inward K^+ current responses induced by nociceptin. However, the lack of selectivity and the low potency ($IC_{50} \approx 10$ μM) of these compounds prevent their use for receptor characterization. Dooley *et al*. (1997) identified 15 compounds having high affinity for the OP_4 receptor from a combinatorial library containing more than 52 million different hexapeptides. When tested in functional assays, however, these peptides showed partial agonist activities (Dooley *et al*., 1997), a property that limits their utility as pharmacological tools. Recently, naloxone benzoylhydrazone (NalBzoH), a non selective opioid receptor ligand (Paul *et al*., 1990), was reported to competitively block some effects of nociceptin in both central (Noda *et al*., 1998; Schlicker *et al*., 1998) and peripheral (Nicholson *et al*., 1998) preparations with similarly low pA_2 values (about 6.3). We have recently reported that the pseudopeptide [Phe$^1\psi$(CH$_2$-NH)Gly^2]nociceptin(1-13NH$_2$ ([F/G]NC(1-13)NH$_2$) is a fairly potent ($pA_2 \approx 7$), selective and competitive OP_4 receptor antagonist (Guerrini *et al*., 1998). Peripherally, the antagonistic properties and the selectivity of action of this compound have been demonstrated *in vitro* and *in vivo* (Bigoni *et al*., 1999; Madeddu *et al*., 1999). However, when tested in central preparations (Butour *et al*., 1998; Calo *et al*., 1998; Okawa *et al*., 1999; Schlicker *et al*., 1998; Xu *et al*., 1998) [F/G]NC(1-13)NH$_2$ shows partial or full agonistic activities. Therefore this molecule is also of limited value for receptor characterization and classification as well as for defining the physiological roles of the NC/OP_4 receptor system in the central nervous system.

In the frame of a structure activity study on nociceptin (Guerrini *et al*., 1999) we have identified new molecules that show OP_4 antagonistic activity in the mouse vas deferens: one of those, [Nphe^1]nociceptin(1-13)NH$_2$ ([Nphe^1]NC(1-13)NH$_2$) is presented in this study by describing results of binding and functional assays performed *in vitro* on recombinant and native OP_4 receptors, as well as results of *in vivo* studies performed in the mouse with the tail withdrawal assay. We demonstrated that (i) [Nphe^1]NC(1-13)NH$_2$ acts as a selective and competitive OP_4 receptor antagonist both at recombinant and native OP_4 sites; (ii) [Nphe^1]NC(1-13)NH$_2$ is also active *in vivo* as it prevents the pronociceptive and antimorphine actions of exogenously applied nociceptin in the tail withdrawal assay, in addition (iii) [Nphe^1]NC(1-13)NH$_2$ induces *per se* a dose dependent, naloxone resistant antinociceptive effect and, at relatively low doses, potentiates morphine induced analgesia.

Methods

Binding experiments

CHO_{OP4} cells were maintained in DMEM:F12 (50 : 50) containing 5% foetal calf serum, 2 mM glutamine, 200 μg ml^{-1} hygromycin B and 200 μg ml^{-1} G418. Cultures were maintained at 37°C in 5%CO_2/humidified air. When confluent, cells were harvested, membranes prepared and used fresh each day as described previously (Okawa *et al*., 1999). All binding assays were performed in 1 ml volumes of Tris-HCl (50 mM), $MgSO_4$ (5 mM) containing 30 μM of the peptidase inhibitors; captopril, amastatin, bestatin, phosphoramidon and bovine serum albumin (0.5%), pH 7.4 for 30 min at room temperature. Incubations contained approximately 2 μg of membrane protein and 3–4 pM [^{125}I]-Tyr^{14}-nociceptin. Non specific binding was defined in the presence of 1 μM nociceptin. Bound and free radioactivities were separated by rapid vacuum filtration using a Brandel cell harvester. Harvester papers (Whatman GF/B) were presoaked in polyethyleneimine (0.5%) to reduce non specific binding and loaded onto the harvester wet.

The binding of [^{3}H]-diprenorphine (approximately 0.5 nM) to CHO_{OP1}, CHO_{OP2}, and CHO_{OP3} membranes was essentially as described previously (Hirst *et al*., 1997; 1998; Smart *et al*., 1997). Briefly membranes were incubated in 1 ml volumes of 50 mM Tris pH 7.4 for 60 min at room temperature. Non-specific binding was defined in the presence of 10 μM naloxone and bound and free radioactivity was separated using a Brandel cell harvester as described above. Where [Nphe^1]NC(1-13)NH$_2$ was included as a displacing ligand the peptidase inhibitor cocktail and BSA were added as described above. As reference compounds DPDPE was included for CHO_{OP1}, norbinaltorphimine for CHO_{OP2} and fentanyl for CHO_{OP3} cells.

Cyclic AMP accumulation experiments

For the measurement of cyclic AMP, whole CHO_{OP4} cells were incubated in 0.3 ml volumes of Krebs-HEPES buffer containing peptidase inhibitors and BSA as described above. In addition 1-isobutyl-4-methylxanthine (1 mM) and forskolin (1 μM) were also included. Concentration response curve to nociceptin was constructed in the absence and presence of 1, 3 and 10 μM [Nphe^1]NC(1-13)NH$_2$. Cells were incubated at 37°C for 15 min after which time the reactions were terminated and cyclic AMP assayed using a protein binding assay as described by Okawa *et al*. (1999).

Electrically stimulated isolated organs

Tissues for *in vitro* studies were taken from male Swiss mice (25–30 g), guinea-pigs (300–350 g), Sprague Dawley rats (300–350 g), and New Zealand albino rabbits (1.5–1.8 kg). The tissues were prepared as described previously (Bigoni *et al*., 1999) and suspended in 10 ml organ baths containing Krebs solution oxygenated with 95% O_2 and 5% CO_2. The temperature was set at 33°C for the mouse vas deferens and at 37°C for the other tissues. A resting tension of 0.3 g was applied to the mouse vas deferens, 1 g to the guinea-pig ileum, rat vas deferens, and rabbit vas deferens and 0.15 g to the guinea-pig renal pelvis. For the experiments on the mouse vas deferens a Mg^{2+}-free Krebs solution was used and for rat vas deferens experiments a Krebs solution containing 1.8 mM $CaCl_2$. Guinea-pig renal pelvis experiments were performed in the presence of indomethacin (3 μM).

The mouse vas deferens, guinea-pig ileum, rat vas deferens, and rabbit vas deferens tissues were continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 ms duration and 0.1 Hz frequency. The electrically evoked contractions were measured isotonically with a strain gauge transducer (Basile 7006) and recorded on a Linseis multichannel chart recorder (model 2005). After an equilibration period of about 60 min the contractions induced by electrical field stimulation were stable; at this time, cumulative concentration response curves to nociceptin or to opioid peptides were performed (0.5 log unit steps). In some experiments, [Nphe^1]NC(1-13)NH$_2$ or naloxone were added to the medium 15 min before performing concentration response curves to the agonists. The guinea-pig renal pelvis was stimulated through two platinum ring electrodes with 100 V square wave pulses of 1 ms duration,

at a frequency of 5 Hz for 10 s. The spontaneous activity and the positive inotropic responses to electrical field stimulation were measured by a Basile isotonic transducer (model 7006) and recorded by a two channel Gemini recorder (model 7070). The experiments commenced following 60 min of equilibration. Four electrical field stimulations were performed in each tissue at 30 min intervals. Agonists were added to the bath 5 min, and antagonists 15 min before the next stimulus. The contractile responses to electrical field stimulation were expressed as % increment of the spontaneous activity of the tissue; the biological effects of the application of agonists or antagonists were expressed as % inhibition of electrical field stimulation-induced contraction.

Tail withdrawal assay

Male Swiss mice weighing 20–25 g were used. The animals were acquired, cared for and used in accordance with the guidelines published in the European Communities Council directives (86/609/EEC). They were housed under standard conditions (22°C, 12 h light-dark cycle) with food and water *ad libitum* at least 2 days before the experiments began. Animals were used only once. I.c.v. injections were made directly into the right lateral ventricle. All experiments were started at 10.00 h. Nociception was assessed using the tail withdrawal assay: the animals were placed in a holder and the distal half of the tail was immersed in water; the withdrawal latency time was measured by an experienced observer blind to drug treatment. A cut off time of 20 s (water temperature at 48°C) or 10 s (water temperature at 55°C) was chosen to avoid tissue damage. Five mice were randomly assigned to each experimental group. Tail withdrawal time was determined immediately before and at 5, 15, 30 and 60 min after i.c.v. injection of 2 μl of saline (control) or of various treatments. In some experiments, naloxone was administered subcutaneously (s.c.) 5 min before i.c.v. injections of saline or [Nphe^1]NC(1-13)NH_2. The time courses of tail withdrawal latency measured in animals subjected to different treatments are shown in the figures. The raw data from each animal were converted to the area under the time × tail withdrawal latency curve (AUC min s^{-1}), as previously described (Calo *et al*., 1998). We calculated the AUC data for the interval of time in which nociceptin induced a clear change of tail withdrawal latencies, namely the 5–30 min period. AUC values obtained under the various treatments were statistically compared by means of one-way ANOVA followed by the Dunnett test for multiple comparisons.

Most of the tail withdrawal experiments were performed with the water temperature set at 48°C; under this experimental condition the tail withdrawal latencies were about 5–6 s, and allowed us to investigate the pronociceptive as well as the antimorphine actions of nociceptin under the same experimental conditions (Calo *et al*., 1998). Thus, we chose the 48°C temperature as it produced a tail withdrawal latency time which can be either reduced (by nociceptin) or increased (by morphine). Under these experimental conditions, we evaluated the effects of the novel ligand [Nphe^1]NC(1-13)NH_2. In addition to this, we also evaluated the actions of [Nphe^1]NC(1-13)NH_2 on morphine induced analgesia. Therefore we set the water temperature to 55°C. Under these experimental conditions the tail withdrawal latencies were always <0.5 s, and we constructed dose response curves to morphine (1–10 nmol range) in the absence and presence of [Nphe^1]NC(1-13)NH_2. For this series of experiments, a cut off time of 10 s was chosen to avoid tissue damage. The analgesic action of i.c.v. administered morphine was compared to that elicited by morphine plus [Nphe^1]NC(1-13)NH_2. The results of these experiments were expressed as percentage of the maximal possible analgesic effect. This was calculated as follows: maximal possible analgesic effect = (latency after drug administration – baseline latency)/(cut-off latency – baseline latency) × 100. The dose of morphine inducing 50% of the maximal possible analgesic effect (D_{50}) was estimated graphically.

Drugs

The peptides used in this study were prepared and purified as described previously (Guerrini *et al*., 1997). [Nphe^1]NC(1-13)NH_2 indicates that the side chain of Phe^1 has been shifted from the C to the N atom in the NC(1-13)NH_2 sequence. Naloxone and indomethacin were from Sigma Chemical Co. (St. Louis, U.S.A.); morphine was from Salars (Como, Italy). DPDPE, fentanyl, captopril, amastatin, bestatin, cyclic AMP, IBMX, nor-binaltorphimine, HEPES and Tris were from Sigma Chemical Co. (Poole, U.K.). Phosphoramidon was from peptide institute (Osaka, Japan). All tissue culture media and supplements were from Gibco (Paisley, U.K.). [^{125}I]-Tyr^{14}-nociceptin (specific activity, 2000 Ci $mmol^{-1}$) was from Amersham (Little Chalfont, U.K.). [2,8-^{3}H]-cAMP (28.4 Ci $mmol^{-1}$) and [15-16-^{3}H]-diprenorphine (58 Ci $mmol^{-1}$) were from NEN DuPont (Boston, MA, U.S.A.). All other standard materials and reagents used were of the highest purity available.

Data analysis and terminology

All data are expressed as means ± s.e.mean of n experiments. Data have been statistically analysed with Student two-tailed t-test or one way ANOVA plus Dunnett test, as specified in table and figure legends. P values less than 0.05 were considered to be significant. The pharmacological terminology used in this study follows the recent IUPHAR recommendations (Jenkinson *et al*., 1995). The agonist potencies are given as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. The E_{max} is the maximal effect that an agonist can elicit in a given preparation. Antagonist potencies are expressed in terms of pA_2, which is the negative logarithm to base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original submaximal response.

Results

In vitro *studies*

Receptor binding and cyclic AMP accumulation in CHO cells The ability of [Nphe^1]NC(1-13)NH_2 to bind to opioid receptors has been evaluated using membranes of CHO cells expressing recombinant mouse OP_1, rat OP_2, rat OP_3, and human OP_4 receptors. As shown in Table 1, [Nphe^1]NC(1-13)NH_2 was essentially inactive at OP_1 and OP_3 sites, where less than 20% displacement (at 10 μM) of [^{3}H]-diprenorphine binding was observed. As internal positive assay controls DPDPE and fentanyl displaced [^{3}H]-diprenorphine with pK_i values consistent with those previously reported (Hirst *et al*., 1998; Smart *et al*., 1997). In contrast to OP_1 and OP_3, [Nphe^1]NC(1-13)NH_2 inhibited [^{3}H]-diprenorphine binding to OP_2 with a pK_i of 5.96. In these cell membranes, nor-binaltorphimine displaced with a pK_i consistent with previous

reports (Hjorth *et al*., 1995). As depicted in Figure 1, the pseudopeptide produced a concentration dependent inhibition of [^{125}I]Tyr^{14}-nociceptin binding to OP$_4$ cell membranes with nonomolar affinity. The shape of the inhibition curve was similar to that obtained with nociceptin; [Nphe^1]NC(1-13)NH$_2$ is however about 100 fold less potent (pK_i 8.39) than the naturally occurring peptide (pK_i 10.12).

We have also examined the effects of [Nphe^1]NC(1-13)NH$_2$ on forskolin-stimulated cyclic AMP formation in CHO$_{OP4}$ cells. [Nphe^1]NC(1-13)NH$_2$ applied in concentrations up to 10 μM, produced a negligible inhibition of forskolin-stimulated cyclic AMP formation ($\alpha^E < 0.15$, not shown). However, when tested against nociceptin, [Nphe^1]NC(1-13)NH$_2$ displaced the concentration response curve of the natural peptide to the right. The antagonistic properties of [Nphe^1]NC(1-13)NH$_2$ were evaluated over the 1 – 10 μM range of concentrations, in order to obtain a Schild plot. As shown in Figure 2 (left panel), [Nphe^1]NC(1-13)NH$_2$ displaced to the right the concentration response curve of NC in a concentration dependent manner, the curves remain parallel to the control, and reach the same maximal effect. Figure 2 (right panel) shows the corresponding Schild plot which was linear ($r = 0.94$) with a slope of 1.11 (not significantly different from unity). The extrapolated pA$_2$ value is 5.96. These data suggest that [Nphe^1]NC(1-13)NH$_2$ acts as a competitive antagonist at the recombinant human OP$_4$ receptor.

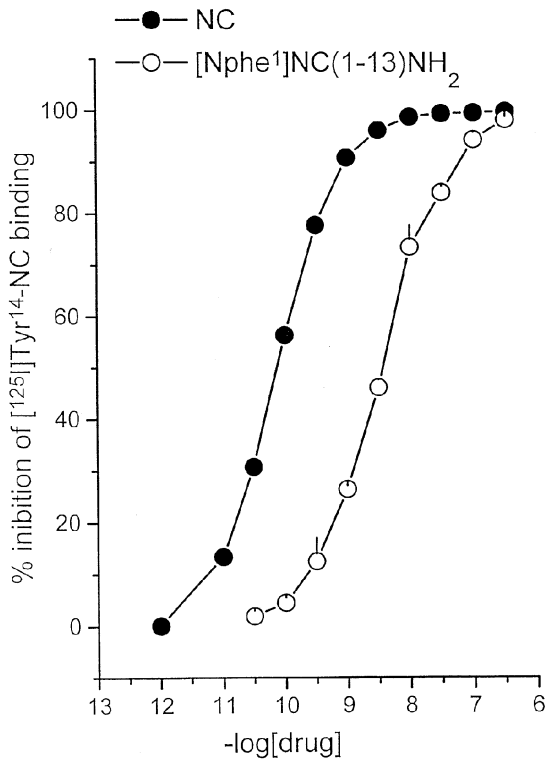

Figure 1 Inhibition of [^{125}I]-Tyr^{14}-nociceptin binding by nociceptin and [Nphe^1]NC(1-13)NH$_2$ to membranes prepared from CHO cells expressing the human OP$_4$ receptor. Points indicate the means and vertical lines the s.e.mean of at least four experiments.

Electrically stimulated isolated tissues

As reported previously (Bigoni *et al*., 1999), nociceptin inhibits the electrically induced contractions of the rat and mouse vas deferens, guinea-pig ileum and renal pelvis. These preparations were used to evaluate the antagonistic properties of [Nphe^1]NC(1-13)NH$_2$ *in vitro*; indeed, the pseudopeptide does not modify the electrically induced twitches of the mouse vas deferens but it displaces to the right the concentration response curve of nociceptin in a concentration dependent (1 – 10 μM) manner (Figure 3, left panel); the curves are parallel to the control, and reach the same maximal effects. Figure 3 (right panel) shows the corresponding Schild plot which is linear ($r = 0.99$) with a slope of 0.95 (not significantly different from unity). The extrapolated pA$_2$ value is 6.04.

Table 1 Receptor binding profile of [Nphe^1]NC(1 – 13)NH$_2$ to recombinant OP$_1$, OP$_2$, OP$_3$ and OP$_4$ receptors expressed in CHO cells

Receptor	*Radioligand*	*[Nphe^1]NC(1–13)NH$_2$* (pK_i)	*Control ligand* (pK_i)
OP$_1$	[^{3}H]-diprenorphine	< 5	DPDPE 8.21
OP$_2$	[^{3}H]-diprenorphine	5.96	NorBNI 9.92
OP$_3$	[^{3}H]-diprenorphine	< 5	fentanyl 8.02
OP$_4$	[^{125}I]-Tyr^{14}nociceptin	8.39	nociceptin 10.12

DPDPE: [D-Pen^{2,5}]enkephalin; NorBNI: nor-binaltorphimine.

Table 2 Characteristics of [Nphe^1]NC(1 – 13)NH$_2$ antagonism of nociceptin actions in electrically stimulated isolated tissues

Preparation	*agonistic activity*	*Residual NC maximal effect*	*Depression of* r	*Schild analysis parameters slope*	*pA$_2$*
Mouse vas deferens	Absent	no	0.99	0.95	6.04
Guinea-pig ileum	Absent	no	0.98	0.94	6.40
Rat ves deferens	Absent	no	0.99	0.86	6.16

Data for the Schild analysis were obtained using [Nphe^1]NC(1 – 13)NH$_2$ at 1, 3, and 10 μM and are means of at least five experiments.

Table 3 Specificity of action of [Nphe^1]NC(1 – 13)NH$_2$ in isolated tissues

Preparation	*Agonist*	*Receptor*	*Naloxone* (pA$_2$)	*[Nphe^1]NC(1 – 13)NH$_2$*
Mouse vas deferens	[DAla^2]deltorphin I	OP$_1$	7.51	inactive
Guinea-pig ileum	dermorphin	OP$_3$	8.82	inactive
Rat vas deferens	etorphine	OP$_?$	8.30	inactive
Rabbit vas deferens	U69593	OP$_2$	8.01	inactive

Inactive: inactive up to 10 μM.

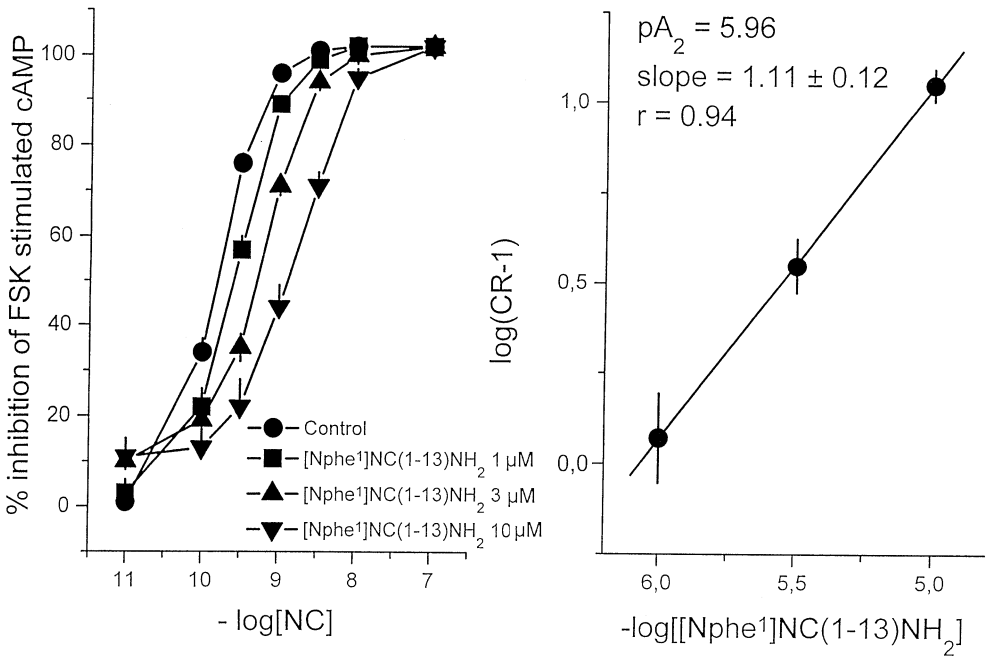

Figure 2 Forskolin stimulated cyclic AMP accumulation in CHO_{OP4} cells. Left panel: concentration response curve to nociceptin in the absence and presence of increasing concentrations of [Nphe^1]NC(1-13)NH_2. The corresponding Schild plot is shown in the right panel. Points indicate the means and vertical lines the s.e.mean of at least five experiments.

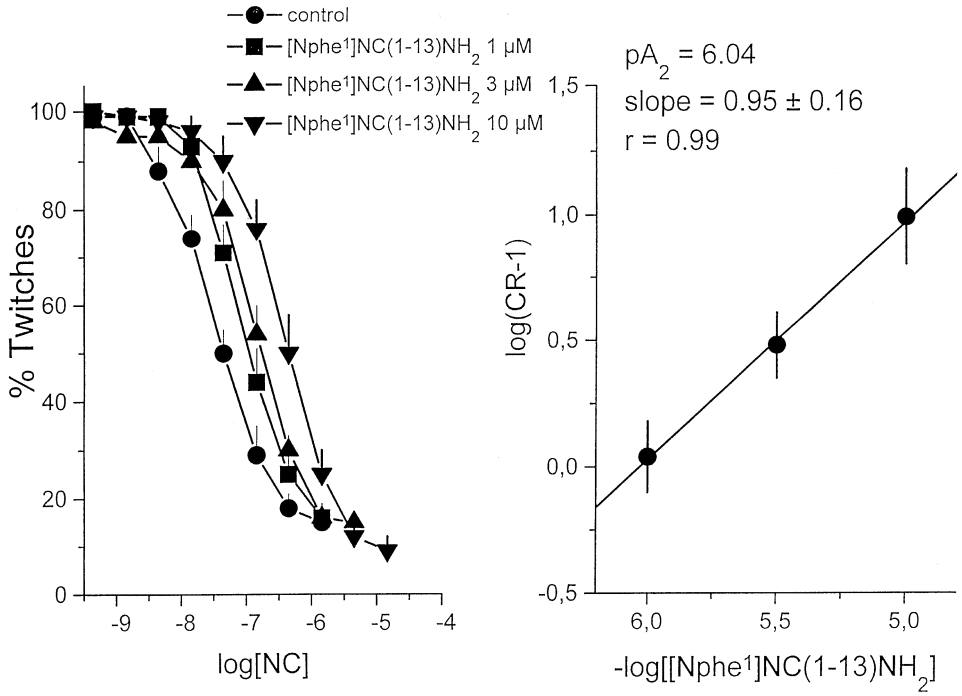

Figure 3 Electrically stimulated mouse vas deferens. Left panel: concentration response curve to nociceptin in the absence and presence of increasing concentrations of [Nphe^1]NC(1-13)NH_2. The corresponding Schild plot is shown in the right panel. Points indicate the means and vertical lines the s.e.mean of at least six experiments.

Similar data obtained in the guinea-pig ileum and the rat vas deferens are summarized in Table 2. [Nphe^1]NC(1-13)NH_2 (contact time 15 min) produced a significant, concentration-dependent, parallel rightward shift of the concentration-response curve to nociceptin in the three preparations without affecting the maximal agonist response (not shown): Schild analysis was compatible with competitive antagonism, yielding slopes not significantly different from unity, and pA_2 values ranging from 6.04 to 6.40 (Table 2). Worthy of mention is the fact that, unlike [F/G]NC(1-13)NH_2 (see Bigoni *et al.*, 1999), [Nphe^1]NC(1-13)NH_2 does not show any residual agonistic activity in these preparations. The antagonistic effect of [Nphe^1]NC(1-13)NH_2 against nociceptin was also evaluated in the guinea-pig renal pelvis, a preparation in which nociceptin inhibits the release of tachykinin from sensory nerves (Giuliani & Maggi, 1996). [Nphe^1]NC(1-13)NH_2 (10 μM) does not modify *per se* the electrically induced contraction of the guinea-pig renal pelvis, but does produce a rightward shift in the concentration response curve to nociceptin (Figure 4) with a pA_2 value of 6.65 (calculated with

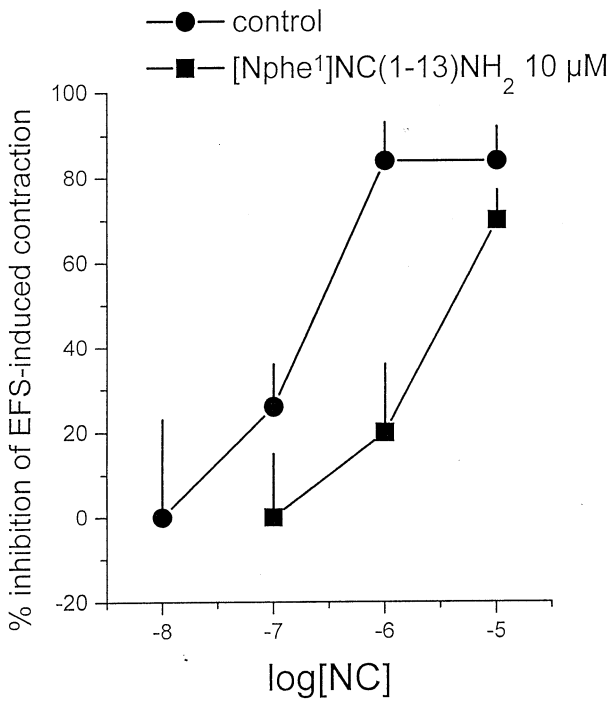

Figure 4 Electrically stimulated guinea-pig renal pelvis. Concentration response curves to nociceptin in the absence and presence of 10 μM [Nphe^1]NC(1-13)NH$_2$ are shown. Points indicate the means and vertical lines the s.e.mean of at least six experiments. E.F.S.: electrical field stimulation.

the Gaddum-Schild equation). In the same tissue, [Nphe^1]NC(1-13)NH$_2$ was found to be inactive against the inhibitory effect elicited by 1 μM dermorphin (control $-87\pm9\%$; 10 μM [Nphe^1]NC(1-13)NH$_2$ $-76\pm10\%$, $n=4$). To further assess its selectivity of action, 10 μM [Nphe^1]NC(1-13)NH$_2$ was tested against the inhibitory action of [D-Ala^2]deltorphin I in the mouse vas deferens, dermorphin in the guinea-pig ileum, etorphine in the rat vas deferens and U69593 in the rabbit vas deferens. [Nphe^1]NC(1-13)NH$_2$ did not produce any change in the concentration response curves to these opioid agonists (Table 3). In contrast, the effects elicited by opioid agonists were all effectively antagonized by naloxone.

In summary our *in vitro* data suggest that this compound is a selective and competitive OP$_4$ receptor antagonist whose *in vivo* actions in the mouse tail withdrawal assay are described below.

In vivo *studies*

Effects of [Nphe^1]NC(1-13)NH$_2$ in the tail withdrawal assay [Nphe^1]NC(1-13)NH$_2$ was tested in the tail withdrawal assay using saline and morphine treated mice under the experimental conditions (water temperature 48°C) and with the experimental protocols described in a recent report (Calo *et al*., 1998). A dose of 30 nmol of [Nphe^1]NC(1-13)NH$_2$ was applied in these experiments based on the ratio of potency nociceptin/[Nphe^1]NC(1-13)NH$_2$ established in *in vitro* experiments.

As shown in Figure 5 (left panel), tail withdrawal reaction time of saline injected mice was stable at values around 5-6 s over the time course of the experiment (AUC: 179 ± 10, $n=6$). Nociceptin (1 nmol) significantly reduced tail withdrawal latency with a maximal effect (about 50% of reduction of the tail withdrawal latency) being obtained at 5 min (AUC: 133 ± 11, $n=6$; $P<0.05$ vs saline). Thirty nmol [Nphe^1]NC(1-13)NH$_2$, administered in the same way as nociceptin, produced a robust antinociceptive effect which peaked at 5 min and progressively decreased during the time course of the experiment (AUC: 292 ± 18, $n=6$; $P<0.05$ vs saline). When nociceptin (1 nmol) and [Nphe^1]NC(1-13)NH$_2$ (30 nmol) are given together either the pronociceptive effect of the natural peptide or the antinociceptive effect of the pseudopeptide are no longer evident (AUC: 195 ± 7, $n=6$). As expected, i.c.v. injection of 1 nmol morphine significantly increases TW latency (AUC: 330 ± 21, $n=5$) (Figure 5, right panel) displaying a maximal analgesic effect at 15–30 min. This analgesic effect of morphine is significantly inhibited by 1 nmol of nociceptin (AUC: 230 ± 16, $n=5$, $P<0.05$ vs morphine). When tested in morphine treated animals, 30 nmol [Nphe^1]NC(1-13)NH$_2$ slightly increased the analgesic effect of the alkaloid (AUC: 357 ± 15, $n=5$) and fully prevented the antimorphine effect of nociceptin (AUC: 279 ± 33, $n=5$).

In a separate series of experiments, the dose dependency and naloxone sensitivity of [Nphe^1]NC(1-13)NH$_2$ induced antinociception was investigated. As shown in Figure 6 (left panel) the antinociceptive effect of [Nphe^1]NC(1-13)NH$_2$ is dose dependent in the 3–30 nmol range (AUC: saline 184 ± 13, [Nphe^1]NC(1-13)NH$_2$ 3 nmol 216 ± 14, 10 nmol 267 ± 40, 30 nmol 359 ± 32 $P<0.05$ vs saline). Naloxone at 3 mg kg^{-1} s.c., a dose which has previously been reported to fully prevent the analgesic action of 1 nmol morphine (Calo *et al*., 1998), does not significantly modify tail withdrawal latencies in saline injected animals (AUC: saline 183 ± 22, naloxone 186 ± 12, $n=6$) and does not affect the antinociceptive effect of 30 nmol [Nphe^1]NC(1-13)NH$_2$ (AUC: 30 nmol [Nphe^1]NC(1-13)NH$_2$ 315 ± 32, plus naloxone 395 ± 12, $n=6$) (Figure 6, right panel).

To further examine the actions of [Nphe^1]NC(1-13)NH$_2$, a dose response curve to morphine was constructed in mice treated in the presence of either saline or 3 nmol [Nphe^1]NC(1-13)NH$_2$. These series of experiments were performed under 'classical' tail withdrawal assay conditions with the water temperature set at 55°C. Under these experimental conditions, tail withdrawal latencies of saline injected animals are always <0.5 s. I.c.v. injections of morphine dose dependently (1–10 nmol) increased tail withdrawal latencies, reaching about 90% of the maximal possible analgesic effect after the highest dose (10 nmol). Coinjection of 3 nmol [Nphe^1]NC(1-13)NH$_2$ (which *per se* does not modify tail withdrawal latencies, data not shown) and morphine results in a leftward shift of the morphine dose response curve, the potency of the alkaloid being 3 fold higher in mice treated with 3 nmol [Nphe^1]NC(1-13)NH$_2$ (D_{50} 1.17 nmol) than in the saline controls (D_{50} 3.95 nmol) (Figure 7, left panel). In a separate series of experiments, using the same experimental conditions, we also tested the effects of 10 nmol [Nphe^1]NC(1-13)NH$_2$: as shown in Figure 7 (right panel) the results are similar to those obtained with 3 nmol of the antagonist (D_{50} saline 1.31 nmol; 10 nmol [Nphe^1]NC(1-13)NH$_2$ 3.17 nmol).

Discussion

This decade has witnessed monumental developments in opioid research including the formal identification of OP$_1$, OP$_2$ and OP$_3$ receptors and the isolation of a new

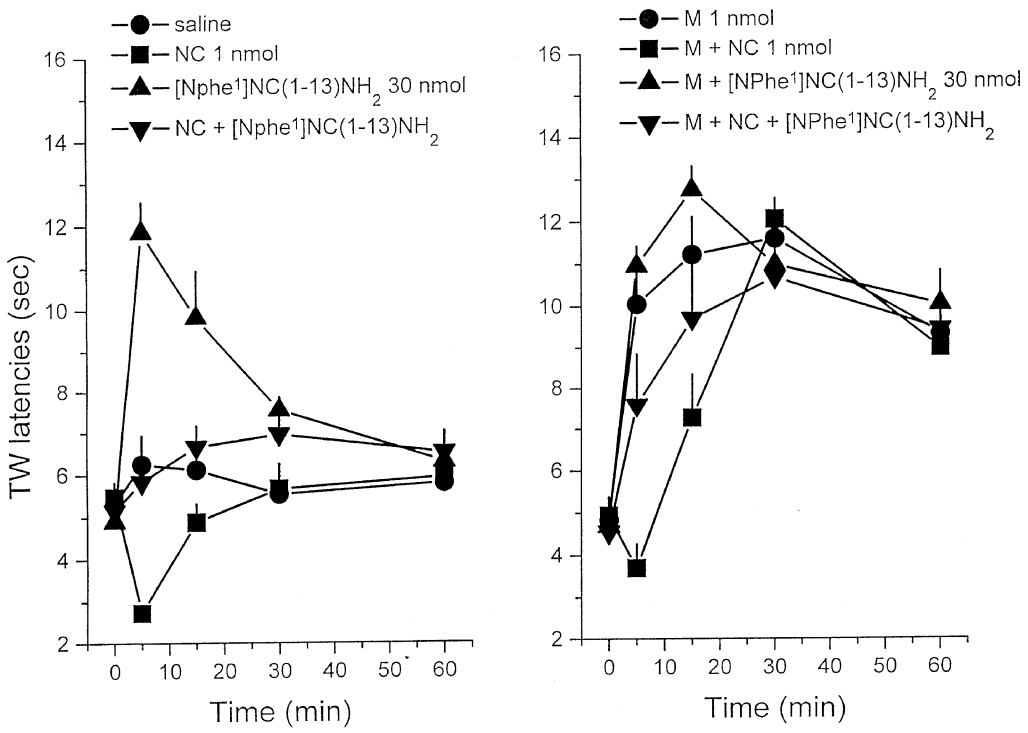

Figure 5 Left panel: Effects of nociceptin (NC, 1 nmol, i.c.v.) and [Nphe^1]NC(1-13)NH$_2$ (30 nmol, i.c.v.) on tail withdrawal latency in mice. Points indicate the means and vertical lines the s.e.mean of at least six experiments. Right panel: Effects of nociceptin (NC, 1 nmol, i.c.v.) and [Nphe^1]NC(1-13)NH$_2$ (30 nmol, i.c.v.) on morphine (M, 1 nmol, i.c.v.) induced analgesia. Points indicate the means and vertical lines the s.e.mean of at least five experiments. AUC calculated from these data were statistically analysed by one-way ANOVA followed by the Dunnett test for multiple comparisons and are presented in the text.

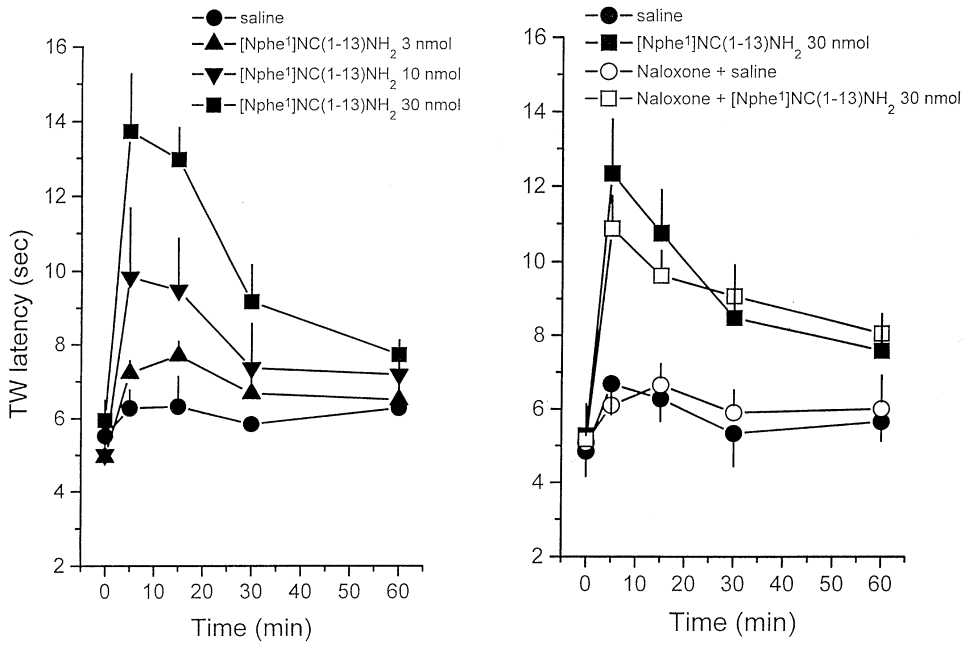

Figure 6 Left panel: Dose response curve to [Nphe^1]NC(1-13)NH$_2$ (3–30 nmol, i.c.v.) on tail withdrawal latency in mice. Points indicate the means and vertical lines the s.e.mean of at least six experiments. Right panel: Effect of naloxone (3 mg kg^{-1}, s.c.) on [Nphe^1]NC(1-13)NH$_2$ (30 nmol, i.c.v.) induced antinociception. Points indicate the means and vertical lines the s.e.mean of at least five experiments. AUC calculated from these data were statistically analysed by one-way ANOVA followed by the Dunnett test for multiple comparisons and are presented in the text.

neuropeptide, nociceptin. This intriguing peptide transmitter interacts with specific cell surface receptors (OP$_4$) to produce a spectrum of effects on nociceptive transmission and animal behaviour. Localization of nociceptin effects to this receptor have, to date, been based largely on the effects of exogenously applied peptide due to the lack of a specific OP$_4$ receptor antagonist. In a comprehensive pharmacological study encompassing both *in vitro* and *in vivo* approaches we describe the characteristics of [Nphe^1]NC(1-13)NH$_2$, a novel truly selective and competitive antagonist for the OP$_4$ receptor.

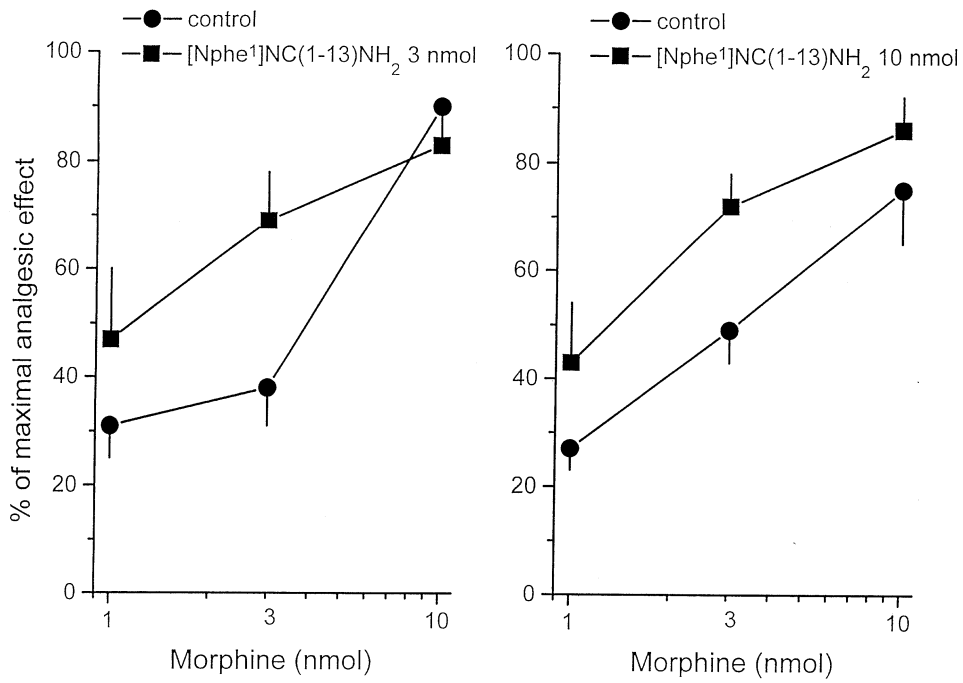

Figure 7 Dose response curve to morphine in mice treated with saline (control), 3 nmol (left panel), or 10 nmol (right panel) of [Nphe^1]NC(1-13)NH$_2$. Points indicate the means and vertical lines the s.e.mean of at least six experiments.

Moreover we suggest that this compound may represent a prototype for a whole new class of analgesic agents with important consequences for the treatment of pain.

We have clearly demonstrated that [Nphe^1]NC(1-13)NH$_2$ binds preferentially to recombinant OP$_4$ receptors where a competitive antagonism of nociceptin inhibition of cyclic AMP formation is observed. This indication of competitive antagonism was confirmed in studies using isolated tissues (vas deferens, ileum, renal pelvis). More importantly in tail withdrawal assays [Nphe^1]NC(1-13)NH$_2$ increases latency alone, reverses the decreased latency produced by nociceptin and potentiates morphine induced antinociception.

Modern pharmacologists have various means at their disposal for evaluating roles and functions of new endogenous agents, especially in the brain. These are antisense oligonucleotides that prevent the expression of either the agent or its receptor, animals whose gene for the agent or the receptor have been knocked out, antibodies directed against the agents, and classical receptor antagonists. These various approaches differ primarily in their kinetics (e.g. the onset and duration of action) which are slow to develop and long lasting for genetic manipulations, intermediate for antisense oligonucleotides, and of rapid onset and short duration for receptor antagonists. The present investigation has been carried out using an OP$_4$ antagonist, [Nphe^1]NC(1-13)NH$_2$, which should allow for the rapid blockade and rapid recovery of nociceptin signalling, and may therefore be less influenced than genetic manipulation by the compensatory mechanisms that are activated to counteract any functional change. Essential requirements for a good antagonist are its selectivity and specificity of action, lack of residual agonistic activity, and, if it is to be used for receptor classification, it should be competitive.

[Nphe^1]NC(1-13)NH$_2$ appears to fulfil all these requirements, since it competes with high affinity with [^{125}I]Tyr^{14}-nociceptin for binding to OP$_4$ but is unable to displace [^{3}H]-diprenorphine from OP$_1$ and OP$_3$ sites and, in these binding assays shows very low affinity for OP$_2$ sites (OP$_4$/OP$_2$ ratio of selectivity: 269). [Nphe^1]NC(1-13)NH$_2$ is therefore selective for OP$_4$: such a selectivity of action has been confirmed by results of bioassays where [Nphe^1]NC(1-13)NH$_2$ has been shown to be inactive against the inhibitory effects exerted by opioid receptor agonists in isolated organs derived from four different species, the guinea-pig, the rat, the mouse and the rabbit, covering OP$_1$-OP$_3$ activity.

[Nphe^1]NC(1-13)NH$_2$ is devoid of agonistic activity: this has been demonstrated in CHO$_{OP4}$ cells where the compound is virtually inactive for inhibition of cyclic AMP accumulation. This contrasts with data obtained in this assay for other putative OP$_4$ antagonists namely [F/G]NC(1-13)NH$_2$ (Butour *et al.*, 1998; Okawa *et al.*, 1999) and NalBzoH (Okawa *et al.*, 1998) which exhibited full and partial agonist activities, respectively. The lack of any agonistic activity of [Nphe^1]NC(1-13)NH$_2$ was also confirmed by bioassay experiments. Indeed, the pseudopeptide did not show any effect *per se* in preparations such as the guinea-pig ileum and especially the rat vas deferens where [F/G]NC(1-13)NH$_2$ produced *per se* a small but consistent inhibition of the electrically induced twitch (Bigoni *et al.*, 1999; Guerrini *et al.*, 1998; Okawa *et al.*, 1999). Moreover, [Nphe^1]NC(1-13)NH$_2$ acts as a pure and competitive OP$_4$ antagonist in the isolated mouse colon where [F/G]NC(1-13)NH$_2$ behaves as a full agonist (Rizzi *et al.*, 1999; Menzies *et al.*, 1999). [Nphe^1]NC(1-13)NH$_2$ exerts a competitive type of antagonism: this has been demonstrated by the parallelism of the concentration response curves of nociceptin, by the fact that maximal effects induced by nociceptin are maintained also in the presence of high concentrations of antagonist, and by the slope of the Schild plot regression line which does not significantly differ from unity in all the various preparations we

studied. Apparent affinities of [Nphe^1]NC(1-13)NH_2, calculated according to Schild, have shown similar values (range 6.0 – 6.4) suggesting that OP_4 from different species belong to the same receptor type. Similar pA_2 values for [Nphe^1]NC(1-13)NH_2 were obtained in the isolated mouse colon and in CHO_{hOP4} cells not only against nociceptin but also against a panel of OP_4 receptor ligands, including [F/G]NC(1-13)NH_2 (Rizzi *et al*., 1999; Hashimoto *et al*., 2000).

The potency of [Nphe^1]NC(1-13)NH_2 is weak, when compared with that of nociceptin or NC(1-13)NH_2 (Bigoni *et al*., 1999; Okawa *et al*., 1999) (about 1/100), and even with that of [F/G]NC(1-13)NH_2 (Bigoni *et al*., 1999; Okawa *et al*., 1999) (about 1/10) while it is close to that of NalBzoH (Okawa *et al*., 1998) (about 1/1). Affinity ratios calculated from binding assays are similar: thus, pK_i of nociceptin is 10.1 vs 8.4 for [Nphe^1]NC(1-13)NH_2 in CHO_{OP4} cell membranes, 8.7 vs 6.9 in mouse brain membranes (Guerrini *et al*., 1999), and 10.3 vs 8.1 in rat cerebral cortex membranes (H. Okawa and D.G. Lambert, unpublished observation). Differences in potency of 10 – 50 folds between binding and functional assay are a common occurrence in peptide pharmacology and have been attributed to varying receptor accessibility in membrane preparations, compared to intact cells and especially intact tissues. However, it is worthy of note that there is a substantial difference between the pA_2 (5.96) and pK_i (8.39) for [Nphe^1]NC(1-13)NH_2 obtained in CHO_{OP4} cell experiments. The pA_2 value in a functional assay would be expected to predict the pK_i in a binding assay. This was not the case and there are several possible explanations for this. Clearly the functional assay (inhibition of cyclic AMP formation) was performed in a physiological buffer and the binding assay was performed in Tris/Mg^{2+} making direct comparison unwise. In addition during the preparation of membranes for binding studies all physiological regulators of ligand binding to the receptor (e.g., guanine nucleotides) will have been washed out.

Worthy of mention is the fact that [Nphe^1]NC(1-13)NH_2 is partially protected from degradation both at the N (by the unnatural Nphe residue) and at the C (by the amide) terminal. This may favour higher potencies *in vivo*, especially in the brain where metabolic degradation may be more important than *in vitro*. This has been taken into account to establish the doses for the *in vivo* assays, where [Nphe^1]NC(1-13)NH_2 was used to characterize a well-documented biological action of nociceptin: its pronociceptive and anti morphine activities in the mouse. In fact, it has repeatedly been shown that nociceptin decreases pain threshold when applied i.c.v. (Meunier *et al*., 1995; Nishi *et al*., 1997; Reinscheid *et al*., 1995) and also reduces the analgesic action of morphine (Mogil *et al*., 1996; Calo *et al*., 1998) in the mouse. These observations led to the suggestion that antagonists of OP_4 receptors may work as centrally acting analgesics.

In the present experiments, [Nphe^1]NC(1-13)NH_2 at 30 nmol mouse^{-1} completely prevented the pronociceptive and antimorphine effects of 1 nmol nociceptin, suggesting that these biological actions are mediated by OP_4 receptor activation. The ability of [Nphe^1]NC(1-13)NH_2 to prevent nociceptin actions *in vivo*, was recently confirmed by Polidori *et al*. (2000) who showed that the pseudopeptide prevents the hyperphagic effect of nociceptin (but not that elicited by neuropeptide Y) in the rat; worthy of mention is the fact that [F/G]NC(1-13)NH_2 acts as an OP_4 agonist in this assay.

In the tail withdrawal assay, [Nphe^1]NC(1-13)NH_2 given alone at low doses potentiated morphine induced analgesia and, at higher doses, caused antinociception. The direct antinociceptive effect of [Nphe^1]NC(1-13)NH_2 was not affected by naloxone which, at the same dose (3 mg kg^{-1}), fully prevented the analgesic action of morphine (Calo *et al*., 1998) thus ruling out the involvement of opioid receptors, at least of the OP_3 type. [Nphe^1]NC(1-13)NH_2 displayed some very weak affinity at OP_2 sites, which may possibly be involved in the analgesic action of the pseudopeptide. This reasonable possibility, however, is unlikely because the dose of naloxone we used (i.e. 3 mg kg^{-1}) is sufficient to block the effects of agonists acting through OP_2 receptors: in fact, (i) 2 mg kg^{-1} naloxone has been reported to antagonize the analgesic effects of U69563 in the mouse abdominal constriction test (Hayes *et al*., 1988); (ii) antinociception, diuresis and inhibition of locomotor activity induced by the selective OP_2 agonist CI-977 in rats were prevented by 1 mg kg^{-1} naloxone (Hunter *et al*., 1990) and (iii) the analgesic action of U69593, U50488 and PD 117302 (all OP_2 agonists) in the rat paw pressure and tail flick tests were antagonized by 1 mg kg^{-1} naloxone (Leighton *et al*., 1988).

Therefore, the lack of sensitivity to naloxone 3 mg kg^{-1} of the antinociceptive effect of [Nphe^1]NC(1-13)NH_2 suggests that this analgesic effect could be attributed to block of the OP_4 receptor. Our data can be interpreted by assuming that, in the brain, endogenous nociceptin tonically controls pain threshold and counteracts the analgesic action of opioids. This view is corroborated by the following findings. Meunier *et al*. (1995) showed that the i.c.v. application of antisense oligonucleotides directed against the OP_4 receptor, induces analgesia in the hot-plate test. This was later confirmed in the formalin test in rats (Zhu *et al*., 1997). Other experiments showed that NalBzoH produces antinociception in wild-type but not in KO_{OP4} mice (Noda *et al*., 1998), again suggesting that the block of OP_4 receptors is associated with analgesia. It has also been reported that the analgesic action of electroacupuncture (which is mediated by endogenous opioids) is strongly potentiated either by antisense oligonucleotides against OP_4 (Tian *et al*., 1997) or by antibodies against nociceptin (Tian *et al*., 1998). In apparent contrast with these data, no alterations of pain threshold or of the analgesic action of morphine have been observed in mice lacking the OP_4 gene (Nishi *et al*., 1997). It must however be kept in mind (as underscored by Nishi *et al*., 1997) that nociceptive pain threshold is probably regulated by several systems, which may compensate for the lack of nociceptin signalling in mutant mice. This idea is also supported by the fact that mice lacking the OP_3 receptor, which is the 'mandatory molecular target for morphine' (Kieffer, 1999), show normal nociceptive thresholds (Matthes *et al*., 1996). As noted previously, compensatory mechanisms may efficiently intervene in chronic conditions such as those produced by gene manipulation. Compensatory mechanisms may be less effective against acute changes than those produced by receptor antagonists.

Collectively our results obtained with [Nphe^1]NC(1-13)NH_2, the first pure OP_4 receptor selective antagonist (together with the evidence present in the literature obtained using different pharmacological tools) suggest that the block of nociceptin signalling in the brain raises pain threshold. Therefore, OP_4 receptor antagonists may represent a new class of pain-killer drugs to be used alone or in combination

with opioids. In this context, the following points are worthy of note: (i) nociceptin does not induce conditioned place preference or aversion (Devine *et al*., 1996), therefore drugs interacting with OP_4 receptors should be devoid of motivational actions and addictive properties (abuse liability); (ii) by potentiating the analgesic actions of opioids, OP_4 antagonists should allow reduction in the doses of opioid used, thus reducing acute side effects associated with opioid therapy (nausea, vomiting, respiratory depression, constipation) and (iii) since nociceptin signalling seems to be involved in the development of tolerance to the analgesic action of morphine (Tian *et al*., 1998; Ueda *et al*., 1997) or endogenous opioids (Koster *et al*., 1999), OP_4 antagonists may be able to reduce the development of morphine tolerance. Experiments are under way to evaluate the effects of chronic treatment with [Nphe^1]NC(1-13)NH_2 in the development of morphine tolerance.

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