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[Nphe^1]nociceptin-(1–13)NH_2 selectively antagonizes nociceptin effects in the rabbit isolated ileum

Leng-Hong Pheng [a], Girolamo Calo [b], Remo Guerrini [c], Domenico Regoli [a,*]

[a] *Institute of Pharmacology of Sherbrooke, Universtity of Sherbrooke, 3001, 12e Avenue Nord, Sherbrooke, Quebec Canada J1H 5N4*
[b] *Department of Experimental and Clinical Medicine, Section of Pharmacology, University of Ferrara, via Fossato di Mortara 19, 44100 Ferrara, Italy*
[c] *Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, via Fossato di Mortara 19, 44100 Ferrara, Italy*

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Abstract

When suspended in vitro in isolated organ baths, segments of the rabbit ileum show a fairly strong and stable spontaneous activity, which derives from the continuous release of acetylcholine and the activation of muscarinic receptors, since the activity is completely eliminated by atropine. Dynorphin A (pEC_{50}: 8.6 ± 0.07), neuropeptide Y and its congener human pancreatic polypeptide (pEC_{50}: 9.40 ± 0.10), and nociceptin (pEC_{50}: 8.08 ± 0.12) dose-dependently inhibit the spontaneous activity through the activation of receptors, which are specifically antagonised respectively by naloxone (pA_2: 7.17 ± 0.12), 2-(naphtalen-1-ylamino)-3-phenylpropionitrile (JCF 104; pA_2: 5.80 ± 0.10), and [Nphe^1]nociceptin-(1–13)NH_2 (pA_2: 6.17 ± 0.19). This last compound, a selective nociceptin-receptor (OP_4) antagonist, inhibits the effect of nociceptin in a competitive manner, as demonstrated by Schild analysis. [Nphe^1]nociceptin-(1–13)NH_2 also antagonizes the effects of other OP_4 receptor ligands such as the full agonist, nociceptin-(1–13)-NH_2, and the partial agonists, [Phe$^1\psi$(CH_2–NH)Gly^2]nociceptin-(1–13)-NH_2 (intrinsic activity (α^E) = 0.5) and Ac-RYYWK-NH_2 ($\alpha^E = 0.5$), with pA_2 values ranged from 5.8 to 6.2. These results indicate that the functional site mediating the inhibitory effect of nociceptin in the rabbit ileum, is pharmacologically identical to the OP_4 sites of other species (mouse, rat, guinea pig, man), since the potencies (pA_2 values) of the pure and competitive antagonist [Nphe^1]nociceptin-(1–13)NH_2 is very similar to the values obtained in the other species. Moreover, the rabbit ileum is one of the few isolated organs that allow classifying compounds, which interact with OP_4 receptors as full agonists, partial agonists, or pure antagonists.

Keywords: Nociceptin; [Nphe^1]nociceptin-(1–13)NH_2; OP_4 receptor; Ileum, rabbit

1. Introduction

The opioid receptor like 1 receptor (Mollereau et al., 1994) (hereafter referred to as OP_4, Hamon, 1998) is a G-protein-coupled receptor, which displays a high structural homology with opioid receptors especially of the OP_2 (κ) type (see, for a review, Meunier, 1997). The cellular actions evoked by OP_4 receptor activation are also similar (if not identical) to those induced by classical opioid receptors (Meunier, 1997). Despite these similarities, the OP_4 receptor does not bind opioid ligands with high affinity and appears to regulate distinct biological functions (Meunier, 1997). The endogenous ligand of the OP_4 receptor has been identified as a novel neuropeptide named nociceptin (NC) (Meunier et al., 1995) or orphanin FQ (Reinscheid et al., 1995). Nociceptin has been shown to act as a selective agonist for OP_4 receptors and to be involved in a variety of physiological processes (Meunier, 1997). However, our knowledge in the nociceptin/OP_4 receptor field is still limited due to the lack of a pure and selective OP_4 receptor antagonist.

The fragment nociceptin-(1–13)NH_2 has been shown to possess full biological activities in a variety of assays in vitro (Calo et al., 1996, 1997; Bigoni et al., 1999; Rizzi et al., 1999a,b) and in vivo (Madeddu et al., 1999; Calo et al., 1998b; Polidori et al., 2000). An analog of this fragment, [*N*-phe^1]nociceptin-(1–13)NH_2 has been shown to exert selective antagonism at OP_4 receptors in all the preparations in which it has been tested so far, namely, several isolated tissues from different species ($pA_2 \approx 6$) (Calo et al., 2000a; Rizzi et al., 1999a) and Chinese hamster ovary cells expressing the human recombinant OP_4

* Corresponding author. Tel.: +1-819-564-5341; fax: +1-819-564-5400.
E-mail address: d.regoli@courrier.usherb.ca (D. Regoli).

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receptors ($pA_2 \approx 6$) (Hashimoto et al., 2000); in vivo, the compound prevents the pronociceptive and antimorphine actions of nociceptin in the mouse tail withdrawal assay (Calo et al., 2000a), the hyperphagic effect in the rat (Polidori et al., 2000), bradycardia and hypotension in the mouse (P. Madeddu, personal communication).

Nociceptin and some fragments and analogs (including nociceptin-(1–13)NH_2, [$Phe^1\psi(CH_2–NH)Gly^2$]nociceptin-(1–13)NH_2, and, more recently, [$Nphe^1$]nociceptin-(1–13)NH_2) were used to characterize nociceptin functional sites in isolated tissues of mouse, guinea pig and rat origin (Bigoni et al., 1999; Calo et al., 1996, 1997, 2000a; Guerrini et al., 1998; Okawa et al., 1999; Rizzi et al., 1999a,b) in cells expressing the human OP_4 receptor (Okawa et al., 1999; Calo et al., 2000a), as well as in receptor binding experiments in guinea pig (Varani et al., 1999), mouse (Varani et al., 1998), and rat (Okawa et al., 1999) brain membranes (see, for a review, Calo et al., 2000b). In the present paper, we identified the isolated rabbit ileum as a nociceptin-sensitive preparation and characterized the OP_4 functional sites expressed in this tissue using the above-mentioned ligands.

2. Methods

2.1. Animals

Albino New Zealand rabbits of either sex weighing 1.5–2.0 kg (Charles River, St. Constant, Québec, Canada) were killed according to the guidelines of the Canadian Council for Animal Care. Segments (10 cm from the coecum) of the ileum were immediately removed and placed in a Tyrode solution of the following composition (mM): NaCl (136.8); KCl (2.6); $MgSO_4 \cdot 7H_2O$ (1.05); NaH_2PO_4 (0.47); $NaHCO_3$ (11.9); $CaCl_2$ (2.0); and glucose (5.5). The tissues were cleared of intestinal content, cut into segments (1.5–2 cm) and attached with a thread so as to leave the lumen open to the bathing solution: they were then suspended in organ bath (10 ml) containing oxygenated (95% O_2 and 5% CO_2) Tyrode solution maintained at 37°C. The segment were stretched to a resting tension of 1 g and allowed to equilibrate in the organ bath for 60 min before starting the experiment. The changes of tension were isometrically recorded with Grass (FT 03C) transducers and displayed on a Grass (Model 7D) polygraph.

Under these experimental conditions, the preparations show marked spontaneous activity (0.8–1.0 g), which is regular and stable for at least 8 h.

2.2. Bioassays

Concentration–response curves to nociceptin and related peptides, dynorphin A and human pancreatic polypeptide were obtained in a noncumulative manner by testing the action of each concentration of agonist and washing the tissue repeatedly for 25–30 min before the next injection. Antagonists were added to the organ bath 15 min before the agonists. The relaxations induced by agonists are expressed as percent of the relaxations of the ileum induced by atropine (1 μM).

2.3. Drugs

Nociceptin and related peptides, as well as Dynorphin A were synthesized in our laboratory as previously described (Calo et al., 1998a; Guerrini et al., 1997). Human pancreatic polypeptide was purchased from Bachem (Torrance, CA, USA). 2-(Naphtalen-1-ylamino)-3-phenylpropionitrile (JCF 104) (Gerald, 1996) was kindly provided by Dr. J.L. Fauchère (Servier, Suresnes, France). Naloxone, atropine, and cabamylcholine were obtained from Sigma (St. Louis, MO, USA). Tetrodotoxin was purchased from ICN (Costa-Mesa, CA, USA). All peptides were dissolved in bidistilled water and kept in concentrated solutions (1 mM) at −20°C until use.

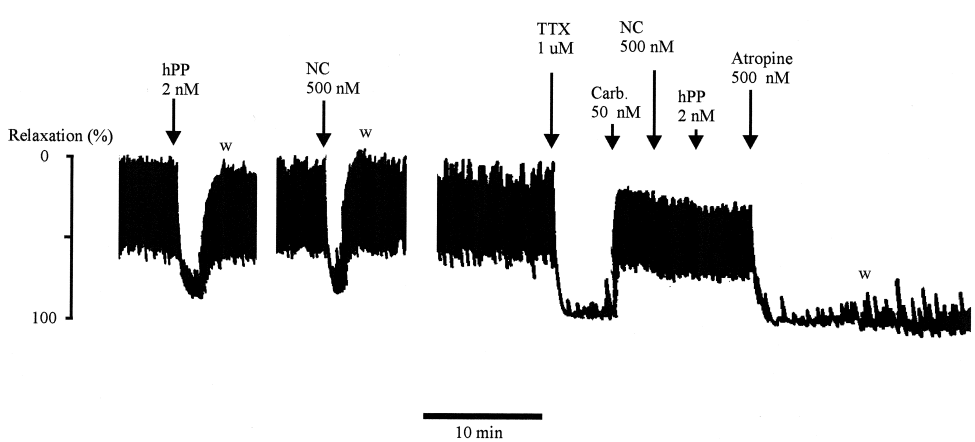

Fig. 1. Tracings obtained with human pancreatic polypeptide and nociceptin on the rabbit ileum. The inhibitory effects of human pancreatic polypeptide and nociceptin are evident on the spontaneous contractions of the tissue, while the peptides are inactive on the contractions induced by carbamylcholine (Carb.) in the tetrodotoxin (TTX) pretreated tissue. Ordinate: relaxation in percent. Abscissa: time in minutes.

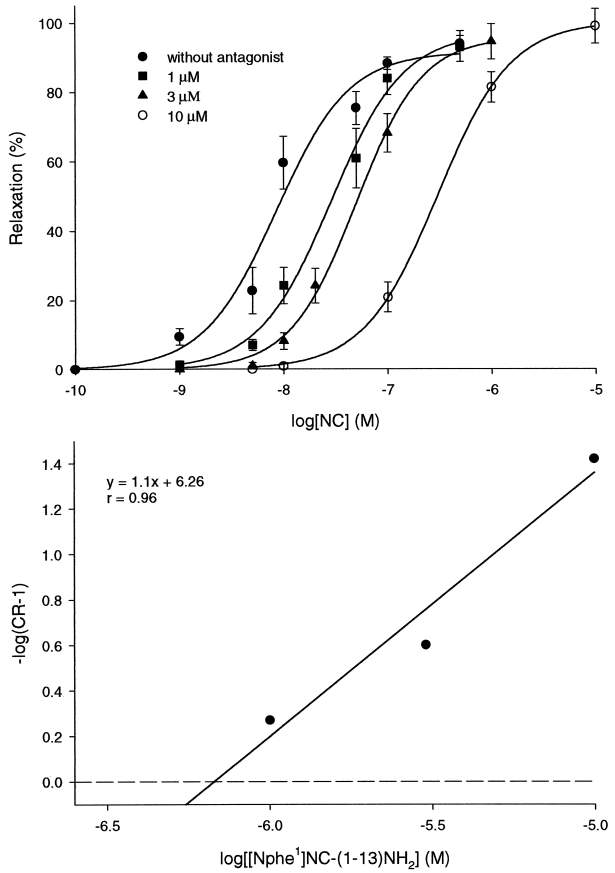

Fig. 2. Top panel: concentration–response curves to nociceptin obtained in the rabbit ileum in the absence and in presence of various concentrations of [Nphe^1]nociceptin-(1–13)NH_2. Bottom panel: Schild plot of [Nphe^1]nociceptin-(1–13)NH_2 against nociceptin. Values are means of at least four separate experiments.

2.4. Data analysis

The data are expressed as means ± S.E.M. Data were statistically analyzed using analysis of variance followed by the Dunnet test for multiple comparisons or with the Student's *t* test as indicated in the tables. *P* values less than 0.05 were considered to be significant. The agonist potencies are given as pEC_{50}, which is the negative logarithm to the 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 tissue. Antagonist affinities are expressed in terms of pA_2, which is the negative logarithm to the base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration in order to elicit the original submaximal response. pA_2 value for [Nphe^1]nociceptin-(1–13)NH_2 against nociceptin was obtained from Schild analysis (Fig. 2), while pA_2 values for [Nphe^1]nociceptin-(1–13)NH_2 against different OP_4 receptor agonists (Table 2), for naloxone against dynorphin A, and for JCF 104 against human pancreatic polypeptide have been calculated using the Gaddum–Schild equation:

$$pA_2 = \log((CR-1)/[\text{antagonist}])$$

assuming a slope value of 1. CR is the ration between the EC_{50} of the agonist obtained in the presence of antagonist and the EC_{50} obtained in the absence of the antagonist.

The pharmacological terminology adopted in this paper is in line with the IUPHAR recommendations (Jenkinson et al., 1995), and also with respect to opioid (Dhawan et al., 1996) and nociceptin (Hamon, 1998) receptor nomenclature.

3. Results

As reported previously, the isolated rabbit ileum shows strong (0.5–0.90 g) and stable (up to more than 8 h) spontaneous activities (Pheng et al., 1997). This spontaneous activity is no longer present when tetrodotoxin (1 μM) or the muscarinic receptor antagonist atropine (0.5 μM) is added to the organ bath (Fig. 1). Cabamylcholine (50 nM) is able to elicit contraction and restore the spontaneous activities in tissues treated with tetrodotoxin (Fig. 1) but not in those incubated with atropine (data not shown).

Nociceptin abolished the spontaneous contractile activity and induces the relaxation of the rabbit ileum, similar to what we previously reported for human pancreatic polypeptide (Fig. 1, see also Pheng et al., 1997). Both peptides, however, fail to inhibit the myotropic activities or relax the contraction produced by carbamylcholine in tissues pretreated with tetrodotoxin (Fig. 1).

The relaxation effect elicited by nociceptin on rabbit ileum is concentration dependent displaying a maximal effect of 94 ± 2% and a pEC_{50} value of 8.08 ± 0.12 (Fig. 2, top panel). [Nphe^1]nociceptin-(1–13)NH_2 up to 10 μM, did not modify per se the ileum spontaneous contractions, but shifted on the right the concentration response curve to nociceptin in a concentration dependent manner (1, 3, and 10 μM), the curves being parallel and reaching the maximum even in the presence of the highest concentration of antagonist (i.e. 10 μM) (Fig. 2, top panel). Schild analysis was compatible with competitive antagonism, yielding a correlation coefficient, slope, and pA_2 values of 0.96, 1.1, and 6.17, respectively.

Table 1
pA_2 values of [Nphe^1]nociceptin-(1–13)NH_2, naloxone, and JCF 104 measured against nociceptin, dynorphin A, and human pancreatic polypeptide in the rabbit isolated ileum
The data are means ± S.E.M. of at least four separate experiments. hPP: human pancreatic polypeptide. All the antagonists were tested at 10 μM against the different agonists.

	Antagonists (pA_2)		
	[Nphe^1]nociceptin-(1–13)NH_2	Naloxone	JCF 104
Agonists			
Nociceptin	6.17 ± 0.19	< 5.0	< 5.0
Dynorphin A	< 5.0	7.17 ± 0.12	< 5.0
hPP	< 5.0	< 5.0	5.80 ± 0.10

Table 2
Maximal effects and pEC_{50} values of four OP_4 receptor ligands recorded in the absence (control) and in the presence of 10 μM [Nphe^1]nociceptin-(1–13)NH_2 in the isolated rabbit ileum
The data are means ± S.E.M. of at least five separate experiments.

	Control		+[Nphe^1]NC-(1–13)NH_2		[Nphe^1]NC-(1–13)NH_2
	E_{max}	pEC_{50}	E_{max}	pEC_{50}	pA_2
Nociceptin	94 ± 2	8.08 ± 0.12	99 ± 5	6.68 ± 0.17*	6.17 ± 0.19
Nociceptin(1–13)NH_2	99 ± 6	8.02 ± 0.09	80 ± 9	7.14 ± 0.01*	5.79 ± 0.01
[F/G]nociceptin-(1–13)NH_2	48 ± 13**	7.38 ± 0.09	28 ± 1	6.37 ± 0.18*	5.90 ± 0.21
Ac-RYYRWK-NH_2	49 ± 5**	8.04 ± 0.05	46 ± 10	7.12 ± 0.07*	5.85 ± 0.10

* Significantly different ($P < 0.05$) compared to the control condition (Student 's t test).
** Significantly different ($P < 0.05$) compared to the E_{max} induced by nociceptin (analysis of variance (ANOVA) plus Dunnet test).

The specificity of action of [Nphe^1]nociceptin-(1–13)NH_2 was evaluated by testing the pseudopeptide in the rabbit ileum against the inhibitory effects elicited by dynorphin A or hPP. As shown in Table 1, [Nphe^1]nociceptin-(1–13)NH_2 at 10 μM did not modify the action of the OP_2 ligand dynorphin A or that of neuropeptide Y receptor agonist human pancreatic polypeptide. The inhibitory effects of these two peptides were, however, selectively antagonized by naloxone and JCF 104 (Pheng et al., 1997), respectively. pA_2 values for [Nphe^1]nociceptin-(1–13)NH_2, naloxone and JCF 104 against nociceptin, dynorphin A, and human pancreatic polypeptide are presented in Table 1.

The action of [Nphe^1]nociceptin-(1–13)NH_2 was also evaluated against several OP_4 receptor ligands, namely, nociceptin-(1–13)NH_2, the smallest fragment maintaining the same potency and efficacy as the natural peptide (Calo et al., 1996), [Phe$^1\psi$(CH_2–NH)Gly^2]nociceptin-(1–13)NH_2, an OP_4 receptor ligand, which behaves as an antagonist, partial agonist or even full agonist depending on the preparation (Bigoni et al., 1999; Calo et al., 1998a,b; Guerrini et al., 1998; Okawa et al., 1999; Rizzi et al., 1999a), and Ac-RYYRWK-NH_2, a high-affinity OP_4 receptor ligand, which was reported to act as a partial agonist at both recombinant and native OP_4 sites (Dooley et al., 1997). These peptides were all able to mimic the inhibitory effect of nociceptin in the rabbit ileum (Table 2). Nociceptin-(1–13)NH_2 behaved as a full agonist showing a maximal effect and a pEC_{50} value (nociceptin-(1–13)NH_2 8.02 ± 0.12; nociceptin 8.08 ± 0.09) similar to that evoked by the natural ligand. [Phe$^1\psi$(CH_2–NH)Gly^2]nociceptin(1–13)NH_2 and Ac-RYYRWK-NH_2 induced maximal effects, which were significantly lower than that induced by nociceptin, thus behaving as partial agonists ($\alpha = 0.51$ and 0.52, respectively) in this preparation. Ac-RYYWK-NH_2 was as potent as the natural peptide, while [Phe$^1\psi$(CH_2–NH)Gly^2]nociceptin-(1–13)NH_2 was about fivefold less potent. [Nphe^1]nociceptin-(1–13)NH_2 (10 μM) antagonized the effects of all these ligands and behaved as a competitive antagonist: indeed, the maximal effects induced by the agonists were not significantly different when measured in the absence or in presence of [Nphe^1]nociceptin-(1–13)NH_2. This new antagonist showed similar pA_2 values (range 5.8–6.2) against the different agonists (Table 2).

4. Discussion

The rabbit ileum is a pharmacological preparation, which can be used both for the study of agents that interact with the smooth muscle and for those that activate or inhibit the myenteric plexus. When suspended in vitro, under the ordinary conditions utilized for pharmacological experiments, the rabbit ileum shows a fairly high spontaneous activity, which remains constant for hours. Treatment of the preparation with tetrodotoxin eliminates the spontaneous contractions and also reduced the basal tonus. The same effects are obtained by blocking the muscarinic receptors by atropine, thus suggesting that the spontaneous activity of this tissue is neurogenic in nature and is probably due to the release of acetylcholine from the myenteric plexus and the subsequent activation of muscarinic receptors in the smooth muscle. The endogenous release of acetylcholine causes an increase of muscle tone in which two components can be separated: a phasic and a tonic one, as shown in the tracing of Fig. 1. Both components can be restored in tetrodotoxin-treated tissues by adding to the organ bath the synthetic agonist carbamylcholine, suggesting that the continuing activation of muscarinic receptors elicits the spontaneous activity of these tissues.

A variety of agents have been shown to reduce the ileum spontaneous activity, while not affecting contractions elicited by carbamylcholine in tissues treated with tetrodotoxin: these results indicate that the receptors for these agents are located prejunctionally. Among these agents, neuropeptide Y and congeners (especially human pancreatic polypeptide, the peptide used in the present experiments, pEC_{50} 9.4 ± 0.1) are the most active (Pheng et al., 1997); however, dynorphin A (pEC_{50} 8.6 ± 0.1) and nociceptin (pEC_{50} 8.08 ± 0.12) show also fairly high potencies. The pEC_{50} value of nociceptin, obtained in the rabbit ileum, is in line with those obtained in other pharmacological preparations of mouse, guinea pig and rat

origin (see the review article by Calo et al., 2000b). This suggests the existence of similar if not identical functional sites in the four species.

OP_4 receptors, expressed in the rabbit ileum, have been characterized, in the present investigation, with a variety of ligands. Nociceptin-(1–13)NH_2 has been reported to be the shortest fragment maintaining the same biological activity as the natural peptide both in vitro (Calo et al., 1996) and in vivo (Calo et al., 1998b): in the rabbit ileum, nociceptin-(1–13)NH_2 mimicked the actions of nociceptin showing similar maximal effects and a slightly higher potency, in agreement with binding data obtained in the mouse (Varani et al., 1998) and guinea pig (Varani et al., 1999) brain membranes. [Phe$^1\psi$(CH$_2$–NH)Gly^2]nociceptin-(1–13)NH_2 is an OP_4 ligand that has been reported to behave as an antagonist (Bigoni et al., 1999; Guerrini et al., 1998; Narita et al., 1999; Okawa et al., 1999) or as a partial agonist (Okawa et al., 1999; Schlicker et al., 1998; Siniscalchi et al., 1999) or even as a full agonist (Butour et al., 1998; Calo et al., 1998b; Okawa et al., 1999; Rizzi et al., 1999a) depending on the preparation/assay. In the rabbit ileum, [Phe$^1\psi$(CH$_2$–NH)Gly^2]nociceptin-(1–13)NH_2 mimicked the effects of nociceptin, however, showing a maximal effect significantly smaller than that evoked by nociceptin: thus, [Phe$^1\psi$(CH$_2$–NH)Gly^2]nociceptin-(1–13)NH_2 acted as a partial agonist in this preparation. Similar results were obtained with Ac-RYYRWK-NH_2. This is a high affinity ligand reported to exert both agonist (Dooley et al., 1997) and antagonist (Berger et al., 1999) activities on OP_4 receptors in different assays. The dual behavior of [Phe$^1\psi$(CH$_2$–NH)Gly^2]nociceptin-(1–13)NH_2 and Ac-RYYRWK-NH_2 may be interpreted assuming that these compounds act as low efficacy agonists whose pharmacological behaviors (antagonist, partial or full agonist) strongly depends on the stimulus/response efficiency characteristic of the preparation under study (see, for a detailed discussion of this topic, Calo et al., 2000b).

[Nphe^1]nociceptin-(1–13)NH_2 applied in increasing concentrations up to 10 μM did not modify the spontaneous activity or the tonus of the rabbit ileum and concentration dependently antagonized the inhibitory effect of nociceptin. Schild analysis suggests that the compound acts as a competitive antagonist. Thus, [Nphe^1]nociceptin-(1–13)NH_2 behaves in this preparation as a pure and competitive OP_4 receptor antagonist. The pA_2 value, calculated for [Nphe^1]nociceptin-(1–13)NH_2 in the present investigation, is similar to those obtained in other nociceptin-sensitive tissues such as the mouse vas deferens and colon, the rat vas deferens, the guinea pig ileum and renal pelvis (range 6.0–6.6) (Rizzi et al., 1999a; Calo et al., 2000a), and to those obtained by studying the inhibitory effect of nociceptin on forskolin stimulated cAMP accumulation in Chinese hamster ovary cells expressing human recombinant OP_4 receptors (Calo et al., 2000a; Hashimoto et al., 2000); therefore, the data obtained with the antagonist demonstrate that no major differences exist between the OP_4 sites from different species. In addition, [Nphe^1]nociceptin-(1–13)NH_2 acts as an effective antagonist also in vivo, where it prevents the pronociceptive and antimorphine action of nociceptin in the mouse withdrawal assay (Calo et al., 2000a), the stimulation of food intake induced by nociceptin in the rat (Polidori et al., 2000), and the hypotensive and bradycardic effects of nociceptin in the mouse (P. Madeddu, personal communication).

In the rabbit ileum, [Nphe^1]nociceptin-(1–13)NH_2 did not modify the inhibitory effects of human pancreatic polypeptide or of dynorphin A; the effects of the two peptides were, however, efficiently antagonized by JCF 104 and naloxone, respectively. Therefore, the antagonist action of [Nphe^1]nociceptin-(1–13)NH_2 is selective for the OP_4 receptors, confirming previous findings of receptor binding experiments performed on membranes of CHO cells expressing the recombinant opioid receptors and of bioassay studies in which the peptide was found to be inactive against the effects elicited by opioid agonists in electrically stimulated isolated tissues (Calo et al., 2000a). Finally, the similar pA_2 values (range 5.8–6.2) obtained with [Nphe^{1}nociceptin-(1–13)NH_2 against a panel of OP_4 ligands demonstrate that all these compounds elicit their effects in the rabbit ileum by activating the same functional site, namely, the OP_4 receptor.

In conclusion, the present investigation suggests that the myenteric plexus of the rabbit ileum expresses OP_4 sites whose activation leads to the inhibition of acetylcholine release and subsequent reduction of spontaneous contractile activity. [Nphe^1]nociceptin-(1–13)NH_2 acts as a pure (devoid of intrinsic activity), selective and competitive antagonist of the OP_4 receptor. Such a unique pharmacological profile (compared to those of the available compounds) makes [Nphe^1]nociceptin-(1–13)NH_2 an essential tool for investigating the biological actions exerted by nociceptin in vitro and in vivo.

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