[Arg^{14},Lys^{15}]Nociceptin, a Highly Potent Agonist of the Nociceptin/Orphanin FQ Receptor: in Vitro and in Vivo Studies

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ABSTRACT
The nociceptin (NC)/orphanin FQ analog, [Arg^{14},Lys^{15}]NC, has been recently demonstrated to behave as a potent agonist at the human recombinant NC receptors (OP$_4$). In this study, we evaluated the pharmacological profile of [Arg^{14},Lys^{15}]NC in vitro on the native OP$_4$ receptors expressed in isolated tissues and in vivo in the locomotor activity and tail-withdrawal assays in mice. On isolated tissues, [Arg^{14},Lys^{15}]NC mimicked the effects of NC, showing similar maximal effects but higher potencies (17-fold in the mouse vas deferens, 10-fold in the rat vas deferens, and about 5-fold in the guinea pig ileum and mouse colon). In these preparations, the effects of [Arg^{14},Lys^{15}]NC were not modified by 1 μM naloxone, although antagonized by the OP$_4$ receptor antagonists [Nphe$^1$]NC(1–13)NH$_2$ (pA$_2$ = 6) and (±)trans-1-[1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one (J-113397) (pA$_2$ = 8). In the rat vas deferens, a cocktail of peptidase inhibitors increased the maximal effects of NC, its analog, and the pEC$_{50}$ of NC (by 4-fold); the potency of [Arg^{14},Lys^{15}]NC was not significantly modified by peptidase inhibitors. In in vivo experiments, [Arg^{14},Lys^{15}]NC mimicked the effects of NC, producing, after intracerebroventricular administration, pronociceptive effects in the tail-withdrawal assay and inhibiting the locomotor activity of the mice. In both assays, [Arg^{14},Lys^{15}]NC was about 30-fold more potent than NC and produced longer lasting effects. Taken together, the present data demonstrate that [Arg^{14},Lys^{15}]NC behaves as a highly potent agonist of the OP$_4$ receptor and is able to produce long-lasting effects in vivo, compared with the natural ligand NC.

Nociceptin (NC)/orphanin FQ is the endogenous ligand of a seven-transmembrane domain G protein-coupled receptor, referred to as OP$_4$. NC and the OP$_4$ receptor are structurally related to opioid peptides and receptors; however, NC does not interact with classical opioid receptors, and the OP$_4$ receptor does not bind any selective opioid receptor ligand (for recent reviews, see Calo' et al., 2000c; Meunier, 2000). It has been demonstrated that NC modulates several biological functions, including pain threshold, morphine analgesia, food intake, anxiety, locomotor activity, memory processes, and cardiovascular, renal, respiratory, and gastrointestinal functions (Calo' et al., 2000c; Meunier, 2000). Further investigations of physiological and pathological roles of the NC/OP$_4$ system require new molecules that potently activate (agonists) or block (antagonists) the OP$_4$ receptor. Identification of such new molecules (at least of peptide nature) should be facilitated by the knowledge of the amino acid residues of the NC sequence that are critical for receptor occupation and activation. Over the last 5 years, several structure-activity relationship studies have been performed on NC (Dooley and Houghten, 1996; Reinscheid et al., 1996; Butour et al., 1997; Guerrini et al., 1997; Calo' et al., 1998a; Guerrini et al., 2000a). These studies suggested that Phe$^1$ and Phe$^4$ represent the critical residues of the message domain of NC (Phe$^1$, Gly$^2$, Gly$^3$, Phe$^4$), which should be involved in receptor binding and activation, whereas the positively charged residues that are present in the address domain of the molecule (Arg$^9$,12, Lys$^9$,13) appear to be crucial for receptor occupation. Indeed, they are expected to interact with the acidic amino acids that are present in the second extracellular loop of the OP$_4$ receptor (for a review, see Guerrini et al., 2000b). The importance of positively charged amino acids was further supported by the fact that the OP$_4$ receptor ligands, identified by screening of peptide combinatorial libraries (Dooley et al., 1997; Becker et al., 1999), are enriched in cationic (Arg/Lys) residues.

Based on these considerations, Okada et al. (2000) recently synthesized analogs in which a Arg-Lys dipeptide was introduced in positions 6–7, 10–11, or 14–15 of NC. Among these

ABBREVIATIONS: NC, nociceptin; J-113397, (±)trans-1-[1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one; ANOVA, analysis of variance.
analogs, [Arg<sup>14</sup>,Lys<sup>15</sup>]<sub>NC</sub> was found to be 3- and 17-fold more potent than NC in binding and functional assays performed on cells expressing the human recombinant OP<sub>4</sub> receptor (Okada et al., 2000).

In the present study, we further characterized the action of [Arg<sup>14</sup>,Lys<sup>15</sup>]<sub>NC</sub> in vitro on native OP<sub>4</sub> receptors expressed in isolated tissues from various species. To pharmacologically characterize the effects of [Arg<sup>14</sup>,Lys<sup>15</sup>]<sub>NC</sub>, we used the selective OP<sub>4</sub> receptor antagonists [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> (Calo' et al., 2000b; Guerrini et al., 2000a), J-113397 (Kawamoto et al., 1999), and the nonselective opioid receptor antagonist naloxone. The effects of [Arg<sup>14</sup>,Lys<sup>15</sup>]<sub>NC</sub> were also evaluated in vivo, in the locomotor activity and in the tail-withdrawal assays after i.c.v. injection of NC, which has been shown to inhibit motility (Rizzi et al., 2001) and evoke pronociceptive effects (Calo' et al., 1998b) in the mouse.

Materials and Methods

In Vitro Studies. Tissues were taken from male Swiss mice (30–35 g), albino guinea pigs (300–350 g), and Sprague-Dawley rats (300–350 g). The mouse vas deferens and colon, the guinea pig ileum, and the rat vas deferens were prepared as previously described (Bigoni et al., 1999; Rizzi et al., 1999). The mouse vas deferens, guinea pig ileum, and rat vas deferens were continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1-ms duration and 0.05 Hz frequency. The electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer [Basile 7006; Ugo Basile Biological Research Apparatus, Comerio (VA), Italy] and recorded with the personal computer-based acquisition system Autotrace 2.2 (RCS, Florence, Italy). 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 NC or [Arg<sup>14</sup>,Lys<sup>15</sup>]<sub>NC</sub> were performed (0.5 log-unit step) in the absence or in the presence of [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> (10 μM), J-113397 (0.1 μM), or naloxone (1 μM), added to the medium 15 min before performing concentration-response curve of the agonists. In one series of experiments, in the rat vas deferens, concentration-response curves to NC and [Arg<sup>14</sup>,Lys<sup>15</sup>]<sub>NC</sub> were performed in the absence and in the presence of a mixture of peptide inhibitors (amastatin, bestatin, phosphoramidon, and captopril) (from Sigma, Milan, Italy) were solubilized in water and stocked at 10 mM concentrations. For in vitro experiments, the compounds were solubilized in physiological solution, and stock solutions (1 mM) were kept at −20°C until use; for in vivo studies, the substances were solubilized in physiological medium just before performing the experiment.

Data Analysis and Terminology. All data are expressed as means ± standard error of the mean of n experiments. Data have been statistically analyzed with Student’s t test or one-way ANOVA followed by the Dunnett test, as specified in table and figure legends; p values less than 0.05 were considered to be significant. The pharmacological terminology adopted in this paper is consistent with the International Union of Pharmacology recommendations (Jenkinson et al., 1995). The agonist potencies were measured as pEC<sub>50</sub>, 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<sub>max</sub> is the maximal effect that an agonist can elicit in a given

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Effects of NC and [Arg<sup>14</sup>,Lys<sup>15</sup>]<sub>NC</sub> in the electrically stimulated mouse vas deferens. Points indicate the means and vertical lines indicate the S.E.M. of at least four experiments.
tissue. In the electrically stimulated tissues, the $E_{\text{max}}$ of agonists is expressed as percentage of inhibition of the control twitch, whereas in the mouse colon it is expressed as percentage of the contraction elicited by 10 μM carbachol. 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. $pA_2$ values of antagonists were calculated by Schild analysis in the mouse vas deferens and, in the other preparations, with the Gaddum Schild equation $pA_2 = -\log(\text{CR-1}/[\text{Antagonist}])$, assuming a slope equal to unity. CR is the ratio of the $E_{\text{50}}$ of an agonist in the presence and in the absence of an antagonist.

Results

In Vitro Studies. NC and [Arg$^{14}$,Lys$^{15}$]NC inhibited in a concentration-dependent manner the electrically induced contraction of the mouse vas deferens with similar efficacy ($E_{\text{max}}$ about 90% inhibition control twitch) but different potencies. Indeed, [Arg$^{14}$,Lys$^{15}$]NC was 17-fold more potent than NC (Fig. 1). The effects of the NC analog were also tested in other preparations, such as the guinea pig ileum and rat vas deferens, in which NC inhibits the twitch response to electrical field stimulation (Bigoni et al., 1999), and in the mouse colon, in which NC induces concentration-dependent contractions (Osinski et al., 1999). In all these tissues, [Arg$^{14}$,Lys$^{15}$]NC elicited similar maximal effects to NC but displayed higher potency by 5-fold in the guinea pig ileum and mouse colon and by 10-fold in the rat vas deferens (Table 1). In these isolated tissues, the kinetics of action of NC and [Arg$^{14}$,Lys$^{15}$]NC were similar; in fact, the effects of both peptides took place immediately after adding the peptide to the bath were rapidly reversible after washing, and could be repeated in the same tissue (data not shown), thus demonstrating lack of tachyphylaxis.

In the mouse vas deferens, the effects of [Arg$^{14}$,Lys$^{15}$]NC were evaluated in the presence of naloxone (1 μM) or of different concentrations of the selective OP$_4$ receptor antagonists [Nphe$^1$]NC(1–13)NH$_2$ (1, 3, 10 μM) and J-113397 (0.03, 0.1, 0.3, 1 μM). Naloxone did not modify the inhibitory effects of [Arg$^{14}$,Lys$^{15}$]NC (Table 2), whereas [Nphe$^1$]NC(1–13)NH$_2$ and J-113397 produced a concentration-dependent rightward shift of the concentration-response curve to [Arg$^{14}$,Lys$^{15}$]NC, without modifying the maximal effects elicited by the peptide (Fig. 2). Schild analysis of these data is compatible with a competitive type of antagonism and yielded $pA_2$ values of 5.86 and 7.85 for [Nphe$^1$]NC(1–13)NH$_2$ and J-113397, respectively (Fig. 2). The antagonists did not show per se any direct effect on electrically induced twitches. Similar data were obtained in the guinea pig ileum, rat vas deferens, and mouse colon in which, however, a single concentration of antagonists was tested; the effects of [Arg$^{14}$,Lys$^{15}$]NC were not modified by 1 μM naloxone, although they were antagonized by 10 μM [Nphe$^1$]NC(1–13)NH$_2$ or by 0.1 μM J-113397. In all preparations, the antagonists did not show any effect per se and did not modify the maximal effects elicited by the NC analog. The $pA_2$ values of [Arg$^{14}$,Lys$^{15}$]NC in different NC-sensitive pharmacological preparations are presented in Table 2.

In the rat vas deferens, a cocktail of peptidase inhibitors (amastatin, bestatin, phosphoramidon, and captopril, all at 30 μM) produced a significant increase in the maximal effects of NC and [Arg$^{14}$,Lys$^{15}$]NC; it also increased NC potency by 4-fold, without significantly modifying the $pE_{\text{50}}$ of [Arg$^{14}$,Lys$^{15}$]NC (Table 3).

In Vivo Studies. [Arg$^{14}$,Lys$^{15}$]NC was administered i.c.v. in mice at doses ranging from 0.01 to 0.3 nmol. Intracerebroventricular injections of [Arg$^{14}$,Lys$^{15}$]NC at 0.01 nmol did not induce any obvious behavioral effects. In contrast, mice injected with 0.1 and 0.3 nmol showed a decrease in locomotor activity and muscular tone (especially in the hindpaws), ataxia, and loss of the righting reflex; however, a normal tail-pinch reflex was maintained. These behavioral effects of [Arg$^{14}$,Lys$^{15}$]NC were very similar to those evoked by high doses (i.e., 10 nmol) of NC, as reported by several investigators (Reinscheid et al., 1995; Devine et al., 1996; Noble and Roques, 1997; Calò et al., 1998b). However, whereas the effects of NC (10 nmol) were fully reversible after 90 min, those elicited by [Arg$^{14}$,Lys$^{15}$]NC (0.3 nmol) were evident for several hours. Indeed, more than 6 h were needed for normal behavior to return in [Arg$^{14}$,Lys$^{15}$]NC-treated animals.

Locomotor Activity Assay. As shown in Fig. 3, mice treated with saline (2 μl/mouse, i.c.v.) displayed a progressive reduction in spontaneous locomotor activity from 195 ± 17 impulses/5 min to 51 ± 8 impulses/5 min during the 30 min of the experiment. [Arg$^{14}$,Lys$^{15}$]NC (0.01–0.3 nmol) caused a dose-dependent reduction of locomotor activity com-

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**TABLE 1**

Biological activities of NC and [Arg$^{14}$,Lys$^{15}$]NC in pharmacological preparations from different species

<table>
<thead>
<tr>
<th>Preparations</th>
<th>NC $E_{\text{max}}$</th>
<th>[Arg$^{14}$,Lys$^{15}$]NC $E_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse vas deferens</td>
<td>-90 ± 2</td>
<td>8.93 (0.09)*</td>
</tr>
<tr>
<td>Mouse colon</td>
<td>48 ± 8</td>
<td>9.12 (0.27)*</td>
</tr>
<tr>
<td>Rat vas deferens</td>
<td>-84 ± 2</td>
<td>8.13 (0.16)*</td>
</tr>
<tr>
<td>Guinea pig ileum</td>
<td>-58 ± 5</td>
<td>8.83 (0.15)*</td>
</tr>
</tbody>
</table>

CL, confidence limits.

* $p < 0.05$ vs. nociceptin (Student’s t test).
pared with saline. This effect was statistically significant for 0.1 and 0.3 nmol (\(p < 0.05\) according to ANOVA followed by the Dunnett test). In the same series of experiments, the effect of 1 nmol of NC was evaluated; as reported previously (Rizzi et al., 2001), at this dose the peptide caused a significant inhibition of locomotor activity only in the first 15 min after i.c.v. injection (data not shown). In the insert of Fig. 3, the dose-response curve of [Arg14,Lys15]NC is compared with that of NC (data taken from Rizzi et al., 2001); [Arg14,Lys15]NC was about 30-fold more potent than the natural ligand.

**Tail-Withdrawal Assay.** Results summarized in Fig. 4 show that tail-withdrawal reaction time of saline-injected mice were stable at values around 4 s over the time course of the experiment. [Arg14,Lys15]NC was ineffective at 0.01 nmol, but it elicited a statistically significant pronociceptive effect that peaked at 15 min and lasted for 30 min at 0.1 nmol. [Arg14,Lys15]NC produced a long-lasting reduction in tail-withdrawal latencies, which was still evident 1 h after the injection of 0.3 nmol (Fig. 4). In some experiments of this series, we tested the effects of 1 nmol of NC and confirmed previous findings (Calo' et al., 1998b); NC caused a significant reduction of tail-withdrawal latencies in the first 15 min after injection (data not shown). Comparison of the effects of [Arg14,Lys15]NC with those elicited by NC under the same experimental conditions (Calo' et al., 1998b) indicate that, also in this assay, [Arg14,Lys15]NC is about 30-fold more potent than the natural ligand and produces longer lasting effects.

**Discussion**

The results presented above demonstrate that [Arg14,Lys15]NC behaves as a highly potent and selective agonist of the OP4 receptor, and its effects in vivo are lasting longer than those of the natural peptide NC. [Arg14,Lys15]NC mimicked the effects of NC by inhibiting in vitro the electrically induced twitches of the mouse vas deferens, rat vas deferens, and guinea pig ileum and by inducing contraction of the mouse colon. In all preparations, the kinetics of action of the two peptides were similar, and their effects were observed immediately after adding the peptide to the bath, were rapidly reversible after washing, and could be repeated several times in the same tissue. In the present study, [Arg14,Lys15]NC displayed higher potencies (5 to 17-fold) than NC, in line with the findings by Okada et al. (2000), who measured an increased binding affinity of 3-fold and a potency increment of 17-fold in the guanosine 5'-3-O-(thio)triphosphate binding assay performed on human embryonic kidney 293 cells expressing the recombinant human OP4 receptor.

To characterize the functional site of action of [Arg14,Lys15]NC in isolated tissues, the effects of the compound were measured in the presence of the classical opioid receptor antagonist, naloxone, and in the presence of two selective OP4 receptor antagonists. [Nphe1]NC(1–13)NH2...
and J-113397. Nphe1 NC(1–13)NH2 is a peptide OP4 receptor ligand, the selectivity of action and antagonist behavior of which have been demonstrated in a wide variety of in vitro and in vivo assays (see Calo’ et al., 2000a). J-113397 is a nonpeptide compound recently identified by investigators at Banyu as a selective antagonist of the recombinant human OP4 receptor (Kawamoto et al., 1999). The antagonist properties and selectivity of action of J-113397 have later been confirmed in vitro on the native OP4 receptor expressed in isolated tissues (Bigoni et al., 2000; Corboz et al., 2000) and in several in vivo studies (Ozaki et al., 2000; Ueda et al., 2000; McLeod et al., 2001). Naloxone (1 μM) was completely inactive against [Arg14, Lys15]NC, although both Nphe1 NC(1–13)NH2 and J-113397 competitively antagonized the effects of the NC analog, showing pA2 values in the range of 5.83 to 6.56 and 7.63 to 8.06, respectively. These pA2 values are similar to those obtained by testing these antagonists against NC (Rizzi et al., 1999; Bigoni et al., 2000; Calo’ et al., 2000b) and therefore demonstrate that the receptor mediating the effects of both NC and [Arg14, Lys15]NC is of the OP4 type.

These data indicate that the addition of two positively charged residues, Arg14 and Lys15, to the sequence of NC leads to increased potency, possibly through an increase in receptor affinity. However, Okada et al. (2000) reported that [Arg14, Lys15]NC binds to OP4 receptor sites expressed in human embryonic kidney 293 cells with a Kᵢ value (0.32 nM) that is only 3-fold higher than that of NC (0.93 nM). Therefore, we performed a series of experiments to investigate if other mechanisms, such as differences in susceptibility to enzymatic degradation, may account for the different potencies of [Arg14, Lys15]NC and NC. Indeed, a cocktail of peptidase inhibitors produced a statistically significant increase in the potency of NC but not of [Arg14, Lys15]NC. The fact that the pEC50 of [Arg14, Lys15]NC is not modified by the inhibitors suggests that the compound is more metabolically stable.
than the natural peptide. This is not surprising because it has been demonstrated that NC can be inactivated by the action of an endopeptidase, which generates the fragments 1–13 and 14–17 (Sandin et al., 1999; Vlaskovska et al., 1999). The addition of positively charged residues (Arg, Lys) in position 14 and 15 may reduce the activity of this enzyme. We therefore suggest that the increased potency of [Arg]^14,Lys]^15)NC may not only result from increased binding affinity but also from lower susceptibility to enzymatic degradation.

[Arg]^14,Lys]^15)NC mimicked the effects of NC also in vivo, by inhibiting the locomotor behavior and by producing pronociceptive effects after i.c.v. administration. Moreover, in both the assays, [Arg]^14,Lys]^15)NC was found to be about 30-fold more potent than NC and to produce long-lasting effects. Indeed, the pronociceptive effects of 0.1 nmol of [Arg]^14,Lys]^15)NC were similar to those elicited by NC at 10 nmol (Calo et al., 1998b), and the same can be said for the motor inhibiting action of [Arg]^14,Lys]^15)NC (0.3 nmol) and NC (10 nmol) (Rizzi et al., 2001). In addition, the pronociceptive effect of [Arg]^14,Lys]^15)NC (0.3 nmol) lasted more than 60 min, whereas that of the natural peptide (at 10 nmol) persisted for only 30 min (Calo et al., 1998b). Finally, the general behavioral effects of [Arg]^14,Lys]^15)NC (decrease in muscular tone (especially in the hindpaws), ataxia, and loss of the righting reflex) were more pronounced and longer lasting than those elicited by NC at 30-fold higher doses. Therefore, the ratio of potency between [Arg]^14,Lys]^15)NC and NC seems to be higher in vivo than in vitro. This further corroborates the hypothesis that the chemical modifications introduced in the [Arg]^14,Lys]^15)NC sequence may confer higher metabolic stability, resulting in the higher potency of the analog in vivo, in which peptidase activity is more relevant.

Taken together, the present data, as well as those of Okada et al. (2000), indicate that [Arg]^14,Lys]^15)NC is the first OP4 receptor agonist more potent than the natural ligand NC. Its effects are long-lasting in vivo. This analog can be considered a novel pharmacological tool for the investigation of the neurobiology of the NC/OP4 receptor system, in particular for clarifying the therapeutic potential of OP4 receptor agonists as anxiolytics (Jenck et al., 1997), antianorectic (Ciccioppo et al., 2001), and antitussives (McLeod et al., 2001), or as novel agents to be used for the treatment of drug abuse (Ciccioppo et al., 2000), cardiovascular (Salis et al., 2000) or renal (Kapusta, 2000) diseases, and urinary incontinence (Lecci et al., 2000; Lazzeri et al., 2001).

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References


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