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In vitro and in vivo pharmacological characterization of the nociceptin/orphanin FQ receptor ligand Ac-RYYRIK-ol

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Abstract

It was recently reported that the hexapeptide Ac-RYYRIK-ol binds with high affinity nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptors and competitively antagonizes N/OFQ actions in the mouse vas deferens assay. Here we further describe the in vitro and in vivo pharmacological features of this NOP receptor ligand. In mouse brain homogenate the degradation half life of Ac-RYYRIK-ol (2.48 min) was significantly higher than that of the parent compound Ac-RYYRIK-NH_2 (1.20 min). In the electrically stimulated mouse vas deferens, Ac-RYYRIK-ol (10–1000 nM) competitively antagonized the inhibitory effect of N/OFQ (pA_2=8.46), while in the isolated mouse colon the hexapeptide mimicked N/OFQ contractile effects thus behaving as a NOP receptor agonist (pEC_{50}=9.09). This latter effect was no longer evident in colon tissues taken from mice knock out for the NOP receptor gene ($NOP^{-/-}$). In vivo in mice, similarly to N/OFQ, Ac-RYYRIK-ol (dose range 0.001–1 nmol) produced: i) pronociceptive effects after intracerebroventricular (i.c.v.) administration and antinociceptive actions when given intrathecally (i.t.) in the tail withdrawal assay; ii) inhibition of locomotor activity and iii) stimulation of food intake after supraspinal administration. Finally, in the forced swimming test, Ac-RYYRIK-ol was inactive per se, but reversed the antidepressant-like effects elicited by the NOP receptor selective antagonist UFP-101 ([$Nphe^1$,Arg^{14},Lys^{15}]N/OFQ-NH_2). Thus, in all these in vivo assays Ac-RYYRIK-ol mimicked the actions of N/OFQ showing however higher potency.

In conclusion, Ac-RYYRIK-ol displayed a complex pharmacological profile which is likely due to the low efficacy agonist nature of this novel ligand of the NOP receptor. The high potency, selectivity of action, and in vivo effectiveness make Ac-RYYRIK-ol a useful pharmacological tool for future studies in the field of N/OFQ and its NOP receptor.

Keywords: Nociceptin/orphanin FQ; NOP receptor; Ac-RYYRIK-ol; Isolated tissue; Behavioral assay; (Mice)

1. Introduction

The cloning of opioid receptors in the beginnings of 90s, was soon followed by the cloning of a novel G-protein coupled receptor that displayed very high homology with opioid receptors but did not bind opioid ligands (Meunier et al., 2000). This receptor was then deorphanized by discovering its endogenous ligand the heptadecapeptide nociceptin/orphanin FQ (N/OFQ) (Meunier et al., 1995; Reinscheid et al., 1995). This was the first successful example of the reverse pharmacology approach (Civelli et al., 1998). The N/OFQ and its N/OFQ peptide receptor (NOP, (Cox et al., 2000)) are widely distributed in the central nervous system, but also in

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the periphery and in the immune system (Mollereau and Mouledous, 2000). Accordingly, N/OFQ elicits a broad range of biological effects such as modulation of pain transmission, of anxiety and response to stress, learning and memory, food intake, locomotor activity. In addition N/OFQ also controls some functions of the renal, cardiovascular, respiratory, and gastrointestinal systems (see for reviews (Calo et al., 2000b; Mogil and Pasternak, 2001)). Thus, the NOP receptor likely represents an interesting molecular target for the development of novel therapeutics (Bignan et al., 2005). The following ligands are considered up to now the best pharmacological tools for investigating the consequences of NOP receptor selective activation or blockage: among NOP receptor agonists the peptide UFP-102 [(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2 (Carra et al., 2005) and the non-peptide Ro 64-6198 (Jenck et al., 2000) and among NOP receptor antagonists the peptide UFP-101 (Calo et al., 2005, 2002) and the non-peptides J-113397 (Ozaki et al., 2000) and SB-612111 (Zaratin et al., 2004). In addition to these compounds, in 1997 a series of hexapeptides of the general formula Ac-RYY-R/K-W/I-R/K-NH_2 having high affinity and selectivity for the NOP receptor were identified from a synthetic combinatorial hexapeptide library containing about 52 million of compounds made considering all the natural amino acids except cystein (Dooley et al., 1997). These hexapeptides were reported to behave as partial agonists at recombinant NOP receptors expressed in Chinese Hamster Ovary (CHO) cells (measured by [^{35}S]GTPγS binding and cAMP accumulation assays) and at native NOP receptor expressed in the mouse vas deferens (estimated by the inhibition of the twitch response induced by electrical field stimulation) (Dooley et al., 1997). These findings were later confirmed and extended by several research groups mainly using two of these hexapeptides namely Ac-RYYRIK-NH_2 and Ac-RYYRWK-NH_2 (Calo et al., 2000a), which were also used as chemical template for performing structure-activity studies. The head to tail cyclization of Ac-RYYRWK-NH_2 produced a drastic decrease in binding affinity (Thomsen et al., 2000) while the N-terminal acylation with a pentanoyl group (Judd et al., 2003) or the replacement of the Tyr^{2,3} residues with (pF)Phe (Judd et al., 2004) led to the identification of high affinity low efficacy NOP receptor ligands. The N-terminal alkylation of the central core YYRW with groups bearing a guanidine function generated a NOP receptor agonist (Ishiama et al., 2001). Moreover the C-terminal addition of a polylysine sequence generated the peptide Ac-RYYRWKKKKKKK-NH_2, named ZP-120, which behaved similarly to the reference compound as a NOP receptor selective partial agonist but displaying higher affinity, metabolic stability and in vivo duration of action (Kapusta et al., 2005b; Larsen et al., 2001; Rizzi et al., 2002). Finally the substitution of the C-terminal amide with an alcoholic function produced Ac-RYYRIK-ol, a NOP receptor ligand that displays high affinity (pK_i 7.91) for NOP receptor sites expressed in the rat brain membrane receptors and competitively antagonized N/OFQ inhibitory effects (pA_2 8.61) in the electrically stimulated mouse vas deferens bioassay (Kocsis et al., 2004).

The present study was aimed to further evaluate the pharmacological profile of Ac-RYYRIK-ol by testing its susceptibility to enzymatic degradation, its in vitro profile in N/OFQ sensitive isolated tissues such as the mouse vas deferens (Calo et al., 1996) and colon (Rizzi et al., 1999) and its effects in vivo in mice in a battery of assays (the tail withdrawal (Calo et al., 1998), locomotor activity (Rizzi et al., 2001), food intake (Rizzi et al., 2005) and forced swimming (Gavioli et al., 2003) tests) routinely used in our laboratories for evaluating ligands interacting with the NOP receptor.

2. Material and methods

2.1. In vitro studies

2.1.1. Estimation of degradation half-life of Ac-RYYRIK-ol, Ac-RYYRIK-NH_2 and N/OFQ in mouse brain homogenates

Brains were obtained from male Swiss mice (Morini, Reggio Emilia, Italy, 25–30 g). The brains were homogenized in 5 vol. (w/v) Tris/HCl (50 mM, pH 7.4, 0 °C) with an ultra-Turrax (Janke Kunkel, Staufen, FRG) using 3×15 s bursts. The supernatant obtained after centrifugation (40 000 ×*g*, 15 min at 4 °C), which contained approximately 8 μg/μl was decanted and used for degradation studies. A 100 μl volume of the peptide (0.6 mg/300 μl Tris) was incubated (final concentration 0.07 μg/μl) with brain supernatant (450 μl) in a total volume of 1 ml containing 50 mM Tris/HCl buffer at pH 7.4. Incubations were performed at 37 °C for varying periods of time up to 10 min. The incubations (100 μl taken for each time) were terminated by addition of trifluoroacetic acid (TFA) 4.5% (200 μl). After centrifugation (40 000 ×*g* for 15 min) an aliquot (100 μl) of clear supernatant was injected in to the Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) column. HPLC was performed on a Kromasil 100-5C18 column (4.6×250 mm) using a Beckman System Gold HPLC system, equipped with a diode array detector. Elution was carried out in a gradient using water (solvent A) and acetonitrile (solvent B) both containing 0.1% of TFA, at a flow rate of 0.7 ml/min. The gradient program used was as follows: linear gradient from 5% to 20% B in 20 min; linear gradient from 20% B to 80% B in 5 min; linear gradient from 80% B to 5% B in 5 min. The eluate was monitored at 220 nm. The degradation half-life ($T_{1/2}$) was obtained by a least-squares linear regression analysis of a plot of peptide pick area versus time, using a minimum of four points.

2.1.2. Mouse vas deferens bioassay

The mouse vas deferens tissues were isolated from male Swiss Mice (20–25 g, Morini, Reggio Emilia, Italy) and prepared accordingly to (Calo et al., 1996). Tissues were suspended in 5 ml organ bath containing Krebs buffer (in mM 118.1 NaCl, 4.7 KCl, 1.8 $CaCl_2{\cdot}2H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 5 Glucose) at 33 °C and incessantly gassed with 5% CO_2 and 95% O_2. A resting tension of 0.3 g was applied. The mouse vas deferens tissues were continuously stimulated

through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 ms duration and 0.05 Hz frequency. Electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006) and recorded with the PC based acquisition system Autotrace 2.2 (RCS, Florence, Italy). Following an equilibration period of about 60 min, the contractions induced by electrical field stimulation were stable and cumulative concentration–response curves to N/OFQ were performed (0.5 log unit steps) in the absence or in the presence (30 min preincubation time) of increasing concentrations of Ac-RYYRIK-ol (10–1000 nM).

2.1.3. Mouse colon bioassay

Segments of mouse (Swiss Mice 20–25 g, Morini, Reggio Emilia, Italy) colon (approximately 1 cm length) were prepared as described previously (Rizzi et al., 1999) to record the isometric smooth muscle contraction. Briefly, the preparations were mounted vertically under 1 g tension in an organ bath (5 ml) containing Krebs at 37 °C and continuously gassed with 5% CO_2 and 95% O_2. Tissues were equilibrated for 60 min with washing every 10 min. For recording the maximal contractile response of the tissues 100 μM carbachol (carbamylcholine chloride) was used. The concentration–response relationships were determined non-cumulatively by adding different concentrations of peptides to the bath every 20 min followed by washing. A separate series of experiments were performed in colon tissues taken from CD1/C57BL6/J-129 $NOP^{-/-}$ and $NOP^{+/+}$ mice (20–25 g). These mice were genotyped by polymerase chain reaction (PCR). Details of the generation and breeding of mutant mice have been published previously (Gavioli et al., 2003; Nishi et al., 1997).

2.2. In vivo studies

2.2.1. Animals

Male Swiss mice weighing 20–25 g were used throughout the studies (Morini, Reggio Emilia, Italy). Mice were handled according to guidelines published in the European Communities Council directives (86/609/EEC) and Italian national regulations (D.L. 116/92). They were housed in 425 × 266 × 155-mm cages (Techniplast, Milan, Italy), eight animals/cage, under standard conditions (22 °C, 55% humidity, 12-h light/dark cycle, light on at 7:00 am) with food (MIL, standard diet; Morini, Reggio Emilia, Italy) and water ad libitum for at least 3 days before experiments began. Each mouse was used only once.

Mice were injected intracerebroventricularly (i.c.v., injection volume 2 μl) under light ether anesthesia (i.e. just sufficient to cause the loss of the righting reflex) using the 'free-hand" technique described by (Laursen and Belknap, 1986). In brief, a 27-gauge needle attached via a polyethylene tube to a 10-μl Hamilton syringe was used for the injection at an angle approximate angle of 45°, at 2 mm lateral to the bregma midline. Each mouse only received one i.c.v. injection. Intrathecal injections (i.t., injection volume 5 μl) were adopted according to the method of (Hylden and Wilcox, 1980). A 28-gauge stainless steel needle attached to a 50-μl Hamilton microsyringe was inserted between the L5 and L6 segment in anesthetized mice.

2.2.2. Tail withdrawal assay

All experiments were started at 10.00 am and performed accordingly to the procedures previously described (Calo et al., 1998). Briefly, the mice were placed in a holder and the distal half of the tail was immersed in water at 48 °C. Withdrawal latency time was measured by an experienced observer blind to the drug treatment. A cut off time of 20 s was chosen to avoid tissue damage. Four mice were randomly assigned to each experimental group and the experiment was repeated at least 3 times. Tail withdrawal latency was determined immediately before and at 5, 15, 30, and 60 min after i.c.v. or i.t. injection of saline (control), and Ac-RYYRIK-ol (0.01–1 nmol).

2.2.3. Locomotor activity test

Experiments were performed starting at 10.00 am, following the procedures previously described (Rizzi et al., 2001). Briefly, the mice were routinely tested 5 min after i.c.v. injection of Ac-RYYRIK-ol (0.01–1 nmol). Locomotor activity was assessed using Basle activity cages, which consist of a four-channel resistance detector circuit which converts the bridges "broken" by the animal paws into pulses that are summed up by an electronic counter every 5 min. Total number of impulses were recorded every 5 min for 60 min. Mice were not accustomed to the cages before drug treatment and the experiment was performed in a quiet and dimly illuminated room. Four mice were randomly assigned to each experimental group and the experiment was repeated at least 3 times.

2.2.4. Food intake assay

The experiments were carried in freely feeding and drinking mice. The experiments took place at 10:00 am. Mice were individually housed in a cage and saline or Ac-RYYRIK-ol (0.001–0.1 nmol) were injected i.c.v. Food intake was measured at 30 and 60 min following drug injection. Food intake was expressed as g/kg of body weight.

2.2.5. Mouse forced swimming test

Experiments were performed following the procedures previously described (Gavioli et al., 2003). The test consists in placing mice, individually, in a Plexiglas cylinders (18.5 cm high, 12.5 cm diameter, water 13.5 cm deep) partially filled with water (24–26 °C), for two swimming sessions: an initial 15-min training session, which was followed, by a 5-min test session 24 h later. The time each animal remained immobile (immobility time) during the 5-min test session was recorded. Animals were judged to be immobile when they ceased struggling/swimming and remained floating motionless in the water, making only those movements necessary to keep their heads above the water line. At the end of each swimming session, the animal

was removed from the cylinder, dried with paper towels, placed in an individual cage for rest and recovered over 15 min, and then returned to its collective home cage. Ac-RYYRIK-ol (0.001–0.1 nmol) was injected i.c.v 5 min before the test. In some experiments, Ac-RYYRIK-ol 0.1 nmol and UFP-101 10 nmol was co-injected i.c.v. 5 min before the test.

2.3. Drugs

The peptides used in this study namely N/OFQ, UFP-101 ([Nphe^{1},Arg^{14},Lys^{15}]N/OFQ-NH$_2$), endomorphin-1, Ac-RYYRIK-NH$_2$ and Ac-RYYRIK-ol were prepared by solid-phase synthesis and purified by HPLC as described previously (Guerrini et al., 1997; Kocsis et al., 2004). All the other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions (1 mM) were prepared in Krebs buffer and saline for the in vitro and in vivo studies, respectively.

2.4. Data analysis and terminology

All data are expressed as means±standard error of the mean of n experiments. Data have been statistically analyzed with the Student's *t*-test or one way analyses of variance (ANOVA) followed by the Dunnett test, as specified in figure legends; *P* values less than 0.05 were considered statistically significant. Curve fitting was performed using PRISM 3.0 (GraphPad Software In., San Diego, U.S.A.). The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations (Neubig et al., 2003). Agonist potencies were expressed 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 tissue/preparation. For the electrically stimulated vas deferens data the E_{max} of agonists is expressed as % of inhibition of the control twitch, while for mouse colon data as percent of the contraction elicited by 100 μM carbachol. Antagonist potencies were expressed as 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 to elicit the original submaximal response (Arunlakshana and Schild, 1959). And were calculated by Schild's linear regression, that correlates the log of concentrations of antagonist (*x* axis) to the log of (CR-1) *y* axis, where CR is the ratio between the EC$_{50}$ (nM) values of the agonist in the presence and absence of antagonist. If the slope of the regression line is not significantly different from the unity, the value of *x* for $y=0$ represents the pA$_2$ value.

In vivo data from studies in mice were analyzed as follows: locomotor activity data were statistically analyzed using the data expressed as cumulative impulses over the 60 min observation period; for tail withdrawal experiments raw data were converted to the area under the time versus tail withdrawal latency curve (AUC min/s). The AUC data for the interval of time (0–60 min) was calculated and these values were used for statistical analysis.

3. Results

3.1. In vitro studies

3.1.1. Degradation half-life of Ac-RYYRIK-ol, Ac-RYYRIK-NH$_2$ and N/OFQ in mouse brain homogenates

The degradation half-life ($T_{1/2}$) of Ac-RYYRIK-ol, Ac-RYYRIK-NH$_2$, and N/OFQ in mouse brain homogenates was obtained by least-squares linear regression analysis of peptide pick area versus time, as shown in Fig. 1. Results of this analysis indicate that Ac-RYYRIK-ol ($T_{1/2}$ 2.48±0.08 min) is much more resistant to the action of the peptidase enzymes contained in the mouse brain homogenate than its parent compound Ac-RYYRIK-NH$_2$ ($T_{1/2}$ 1.20±0.03 min) and similar to that of the naturally occurring ligand N/OFQ ($T_{1/2}$ 3.30±0.02 min).

3.1.2. Mouse vas deferens bioassay

In the mouse vas deferens, N/OFQ inhibited the twitch response to electrical field stimulation in a concentration dependent manner showing an E_{max} of 90±5% and a pEC$_{50}$ of 7.60 (Fig. 2). In this preparation Ac-RYYRIK-ol was completely inactive at 10 and 100 nM while at 1 μM it produced a slight inhibition of the electrically induced twitch in 5 out of 8 tissues. As displayed in Fig. 2, left panel, Ac-RYYRIK-ol tested over the concentration range of 10–1000 nM shifted the concentration response curve to N/OFQ to the right in a concentration dependent manner. Curves obtained in the presence of Ac-RYYRIK-ol were parallel to the control and reached similar maximal effects even in the presence of the highest concentration of compound. The corresponding Schild plot was linear ($r=0.97$) with a slope not significantly different from unity yielding a pA$_2$ value of 8.46 (Fig. 2, right panel).

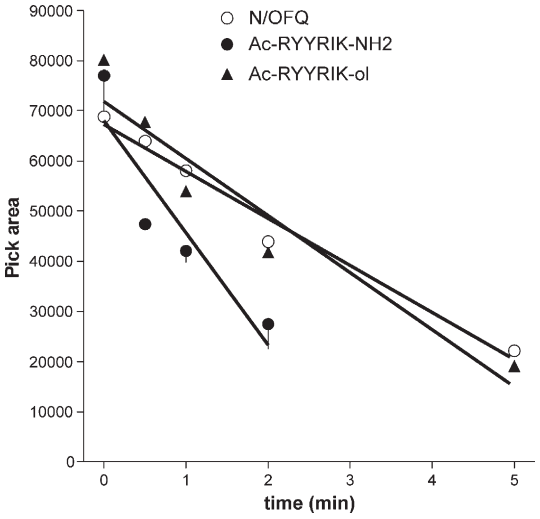

Fig. 1. Degradation half-life ($T_{1/2}$) of N/OFQ, Ac-RYYRIK-NH$_2$ and Ac-RYYRIK-ol in mouse brain homogenates obtained by least-squares linear regression analysis of peptide pick area versus time.

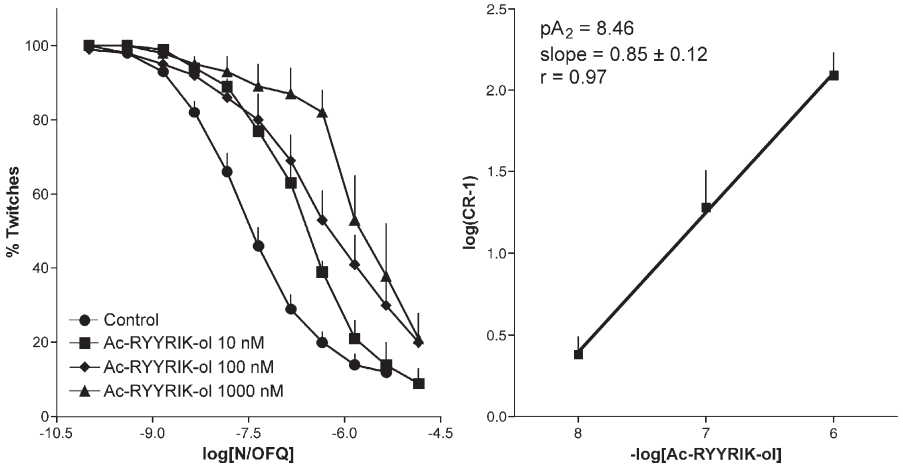

Fig. 2. Effects of N/OFQ and Ac-RYYRIK-ol in the electrically stimulated mouse vas deferens. Left panel: concentration response curves to N/OFQ obtained in the absence (control) and presence of increasing concentrations of Ac-RYYRIK-ol (10–1000 nM). The corresponding Schild plot is shown in the right panel. Points indicate the means and vertical lines the standard error of mean (S.E.M.) of at least 4 separate experiments.

3.1.3. Mouse colon bioassay

In the isolated mouse colon, N/OFQ evoked concentration dependent contractile effects showing a pEC_{50} of 8.80 and an E_{max} of 32.2±1.2%. This effect was mimicked by Ac-RYYRIK-ol which displayed similar maximal effects (28.4±1.3%) but with 2 fold higher potency (pEC_{50} 9.09) (Fig. 3, left panel). The effects of Ac-RYYRIK-ol were also investigated in colon tissues taken from $NOP^{+/+}$ and $NOP^{-/-}$ mice. 100 nM of N/OFQ and Ac-RYYRIK-ol produced a contraction of colon tissues of $NOP^{+/+}$ mice amounting to 37±7% and 32±5% of contraction induced by 100 μM of carbachol, respectively. These two peptides were found completely inactive when tested in tissues taken from $NOP^{-/-}$ mice (Fig. 3, right panel). On the contrary, the selective MOP receptor agonist endomorphin-1 (tested at 1 μM) produced similar contractile effects in tissues from $NOP^{+/+}$ and $NOP^{-/-}$ animals (Fig. 3, right panel).

3.2. In vivo studies

3.2.1. Tail withdrawal assay

I.c.v. injection of 0.01 nmol of Ac-RYYRIK-ol in mice did not induce any effect on gross behavior. In contrast, mice treated with 0.1 and particularly with 1 nmol Ac-RYYRIK-ol displayed a decrease in locomotor activity, ataxia and loss of the righting reflex, similarly to what was observed and reported by various groups after the i.c.v. injection of high doses (>1 nmol) of N/OFQ (Calo et al., 1998; Reinscheid et al., 1995; Rizzi et al., 2001). However, whilst the effects of N/OFQ on gross behavior appeared immediately after i.c.v. injection, those produced by Ac-RYYRIK-ol were slower to develop becoming evident only after 15–20 min. No effects were observed in the gross behavior of the mice after i.t. injection of Ac-RYYRIK-ol.

In the tail withdrawal assay, the i.c.v. injection of saline did not modify tail withdrawal latencies which remained stable

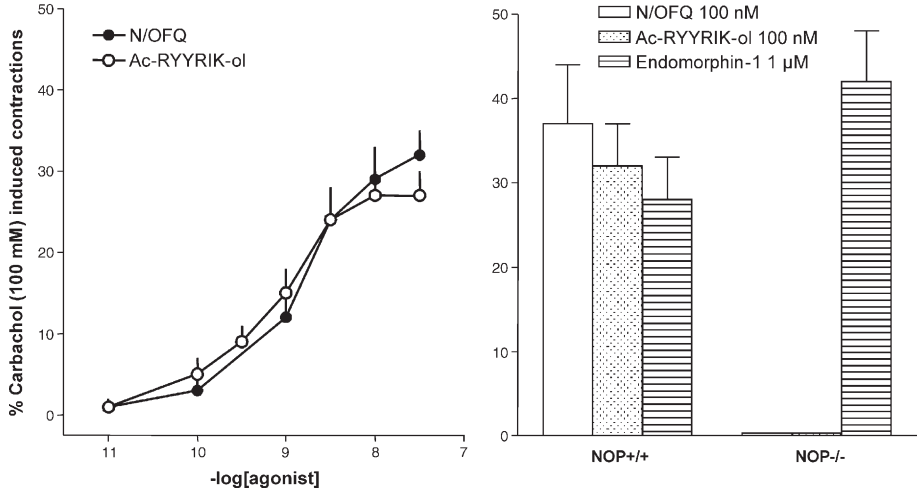

Fig. 3. Effects of N/OFQ and Ac-RYYRIK-ol in the isolated mouse colon. Left panel: concentration response curve to N/OFQ and Ac-RYYRIK-ol in tissues taken from male Swiss mice. Right panel: effect of single concentrations of N/OFQ, Ac-RYYRIK-ol, and EM-1 on mouse colon tissues taken from $NOP^{+/+}$ and $NOP^{-/-}$ mice. Points indicate the means and vertical lines the S.E.M. of at least 3 separate experiments.

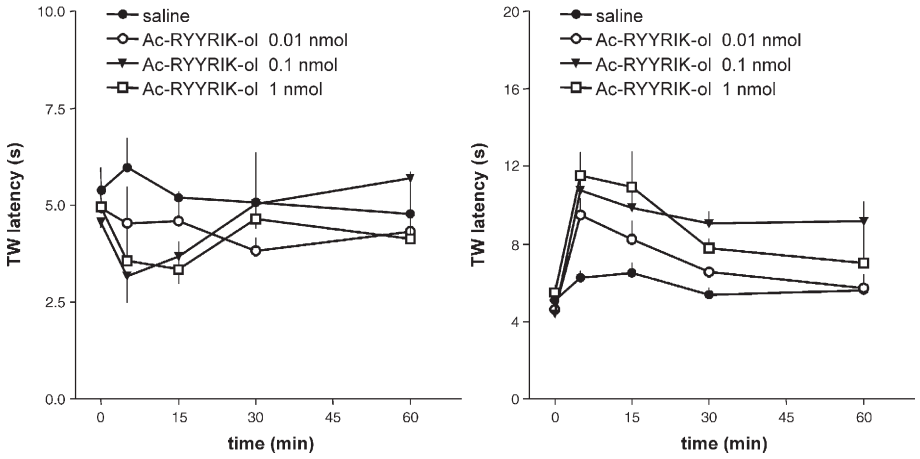

Fig. 4. Effects of Ac-RYYRIK-ol in the mouse tail withdrawal assay. Left panel: dose response curve to Ac-RYYRIK-ol (0.01–1 nmol) injected i.c.v. (AUC(0–60): saline 309±19; 0.01 289±25; 0.1 nmol 256±48; 1 nmol 248±77). Right panel: dose response curve to Ac-RYYRIK-ol (0.01–1 nmol) injected i.t. (AUC(0–60): saline 346±23; 0.01 419±13; 0.1 nmol 556±44*; 1 nmol 518±46*, *$P<0.05$ according to ANOVA followed by the Dunnett's test). Points indicate the means and vertical lines the S.E.M. of 4 experiments.

around 5 s for the time course of the experiments (Fig. 4, left panel). Ac-RYYRIK-ol at the dose of 0.01 nmol failed to alter the animal response while at 0.1 and 1 nmol the compound produced a reduction of tail withdrawal latencies which peaked at 5–15 min and returned to control values after 30 min from the i.c.v. injection. However, AUC (0–60 min) data for 0.1 (256±48) and 1 nmol (248±77) Ac-RYYRIK-ol compared to saline (309±19) failed to reach statistical significance (Fig. 4, left panel).

In the same assay, the i.t. injection of saline did not modify tail withdrawal latencies (Fig. 4, right panel). The i.t. administration of Ac-RYYRIK-ol at the dose of 0.01 nmol produced a slight increase in tail withdrawal latencies. At higher doses (i.e. 0.1 and 1 nmol) the peptide evoked a clear and robust antinociceptive effect which peaked at 15 min and did not return to control values even after 60 min (Fig. 4, right panel).

3.2.2. Locomotor activity test

In the locomotor activity assay, mice injected with saline (2 μl/mouse, i.c.v.) displayed a progressive reduction of spontaneous locomotor activity from 255±6 to 84±7 impulses/5 min during the 60 min time course of the experiment (Fig. 5, left panel). Ac-RYYRIK-ol administered in the 0.01–1 nmol range, caused a dose dependent reduction of locomotor activity compared to saline injected animals (Fig. 5; left panel). With the highest dose of peptide (i.e. 1 nmol) the locomotor behavior of the animals was virtually suppressed. The motor inhibiting action of Ac-RYYRIK-ol was statistically significant at 0.1 and 1 nmol doses (Fig. 5, right panel).

3.2.3. Food intake assay

In food intake studies, the i.c.v. injection of 0.001 nmol of Ac-RYYRIK-ol did not modify food intake of sated mice

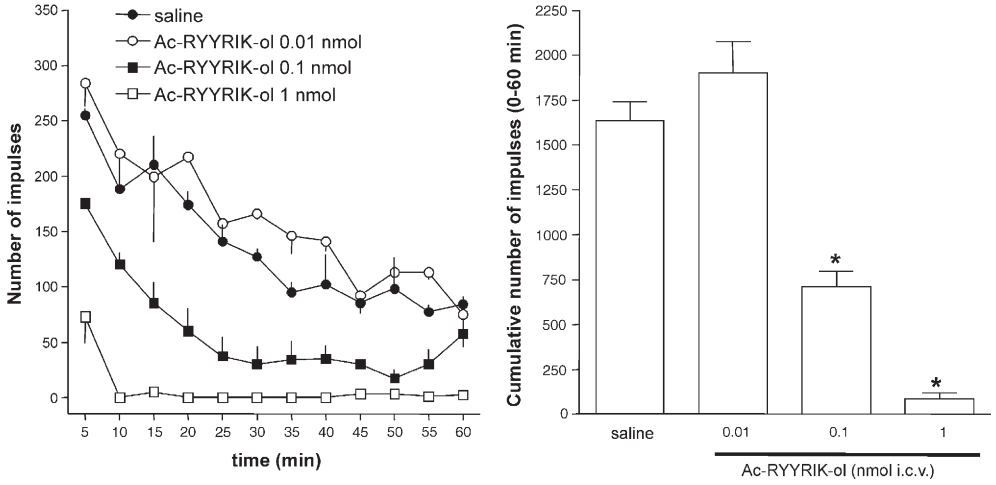

Fig. 5. Effects of Ac-RYYRIK-ol in the locomotor activity in mice. Left panel: dose response curve to Ac-RYYRIK-ol (0.01–1 nmol) injected i.c.v. Points indicate the means and vertical lines the S.E.M. of 4 separate experiments. Right panel: Data expressed as cumulative impulses over 60 min were used for statistical analysis. *$P<0.05$ vs. saline, according to ANOVA followed by the Dunnett's test.

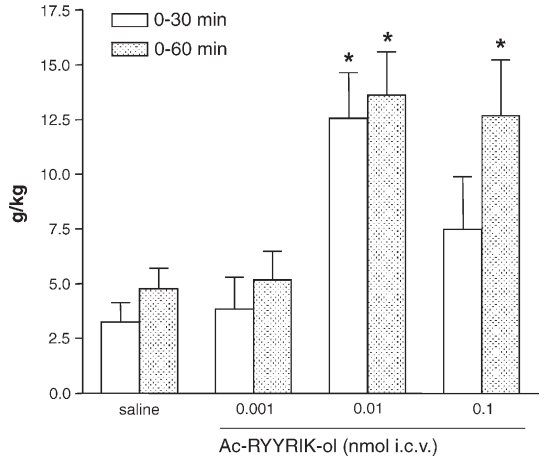

Fig. 6. Cumulative 30 and 60 min food intake following i.c.v. injection of saline or Ac-RYYRIK-ol (0.001, 0.01 and 0.1 nmol). Values are means ± S.E.M. of 12–15 animals. Ordinate: grams/kg body weight. *$P<0.05$ vs. saline according to ANOVA followed by the Dunnett's test.

(Fig. 6). On the contrary, when the peptide was administered at 0.01 and 0.1 nmol it produced a statistically significant stimulation of food intake (Fig. 6).

3.2.4. Mouse forced swimming test

In the forced swimming test, mice treated i.c.v. with saline display an immobility time of 190 ± 17 s (Fig. 7, left panel). Ac-RYYRIK-ol given i.c.v. in the dose range of 0.001–0.1 did not induce any significant modification of this behavioral parameter (Fig. 7, left panel). Conversely, as shown in the Fig. 7, right panel, the NOP receptor antagonist UFP-101 at 10 nmol produced a statistically significant reduction of immobility time (73 ± 19 s). This effect of UFP-101 was no longer evident when the peptide was co-administrated with 0.1 nmol of Ac-RYYRIK-ol.

4. Discussion

The novel NOP receptor ligand, the hexapeptide alcohol Ac-RYYRIK-ol, has been characterized pharmacologically in vitro and in vivo. In vitro in mouse tissues, Ac-RYYRIK-ol antagonized N/OFQ effects in the vas deferens while it mimicked N/OFQ action in the colon; in vivo the peptide consistently behaved as a NOP receptor agonist mimicking the supraspinal pronociceptive, orexigenic, and motor inhibiting actions and the spinal antinociceptive effects of N/OFQ. In addition, Ac-RYYRIK-ol, similar to that of N/OFQ, reversed the antidepressant like effects of the NOP receptor selective antagonist UFP-101. Thus, our results confirm and extend previous findings demonstrating that Ac-RYYRIK-ol displays a complex pharmacological profile which is likely due to the low efficacy agonist nature of this novel ligand of the NOP receptor.

The hexapeptide Ac-RYYRIK-ol was recently identified in the frame of a structure–activity study (Kocsis et al., 2004) on one of the Dooley's hexapeptides, Ac-RYYRIK-NH_2 (Dooley et al., 1997). Briefly the C-terminal carboxyamide residue (–$CONH_2$) was reduced to a hydroxymethylene yielding a reduced primer alcohol like C-terminal end. In receptor binding experiments performed on crude rat brain membranes Ac-RYYRIK-ol displaced [^{3}H]N/OFQ-NH_2 but not [^{3}H]naloxone binding indicating that the ligand selectively recognizes NOP receptor sites (Kocsis et al., 2004). Interestingly, while in binding experiments Ac-RYYRIK-ol displayed a value of affinity (pK_i 7.92) lower than that of the parent compound Ac-RYYRIK-NH_2 (pK_i 8.25), in functional experiments performed in the mouse vas deferens, where both compounds competitively antagonized N/OFQ inhibitory effects, the rank order of potency was opposite: Ac-RYYRIK-ol (pA_2 8.61) > Ac-RYYRIK-NH_2 (pA_2 7.55) (Kocsis et al., 2004). In the present investigation we confirmed the high antagonist potency of Ac-RYYRIK-ol in the mouse vas deferens (pA_2 8.46) and demonstrated that the two peptides display a different susceptibility to enzymatic degradation. In fact the degradation half life in mouse brain homogenates of Ac-RYYRIK-ol ($T_{1/2}$ 2.48 min) was approximately double than that of Ac-RYYRIK-NH_2 ($T_{1/2}$ 1.20 min) and similar to that of N/OFQ ($T_{1/2}$ 3.30 min). The higher metabolic stability of Ac-RYYRIK-ol compared to Ac-RYYRIK-NH_2 may be relevant for interpreting the discrepant results obtained in binding and bioassay experiments. The peptidase activity is likely to be more effective (and relevant in determining ligand

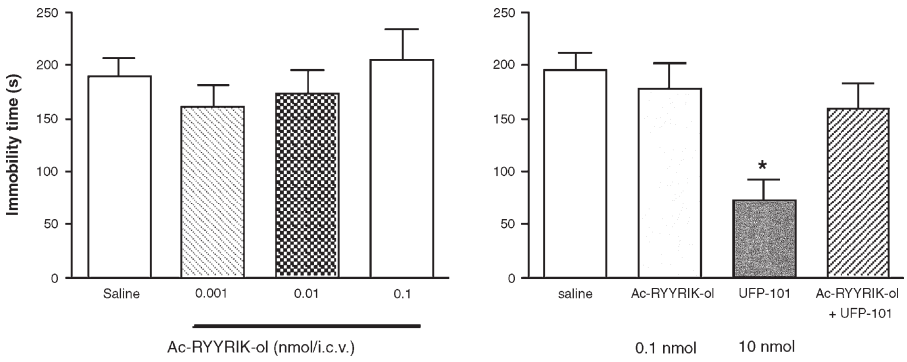

Fig. 7. Effects of Ac-RYYRIK-ol in the mouse forced swimming test. Left panel: dose response curve to Ac-RYYRIK-ol (0.001–0.1 nmol) injected i.c.v. Right panel: effects of 0.1 nmol Ac-RYYRIK-ol and 10 nmol UFP-101 injected alone and co-injected on immobility time of Swiss mice submitted to the test. Values are means ± S.E.M. of 9–13 animals. *$P<0.05$ vs. saline according to ANOVA followed by the Dunnett's test.

potency) in membranes, due to the different cytosolic peptidases in the membrane homogenate, than in intact tissues. Therefore the opposite rank of potency of Ac-RYYRIK-ol and Ac-RYYRIK-NH_2 obtained in binding and bioassay experiments might be related to their different susceptibility to peptidase actions.

In mouse colon experiments Ac-RYYRIK-ol mimicked N/OFQ contractile effects thus behaving as a NOP receptor agonist. The contractile effects of the hexapeptide were no longer evident in tissues taken from $NOP^{-/-}$ mice, demonstrating that, similar to those of N/OFQ (present results and (Di Giannuario et al., 2001)), they derive from the exclusive activation of receptors of the NOP receptor type. Since a supramaximal concentration of Ac-RYYRIK-ol was used in these studies, these findings also corroborate the results obtained in binding experiments (Kocsis et al., 2004) demonstrating that Ac-RYYRIK-ol is highly selective for NOP receptors.

The different pharmacological activity of Ac-RYYRIK-ol at NOP receptors expressed in the mouse vas deferens (antagonist) and colon (agonist) is not completely surprising. In fact, very similar results were reported not only for the parent compound Ac-RYYRIK-NH_2 but also for the NOP receptor ligand [$Phe^1\psi(CH_2–NH)Gly^2$]N/OFQ(1–13)–NH_2: these molecules behaved as NOP receptor antagonists in the vas deferens assay (Guerrini et al., 1998; Kocsis et al., 2004; Mason et al., 2001) and in the GTPγ[^{35}S] binding assay performed in rat brain membranes and mouse brain sections (Berger et al., 2000; Mason et al., 2001), while as NOP receptor agonists in the mouse colon (Rizzi et al., 1999). Interestingly enough, it has been demonstrated that the pharmacological profile of [$Phe^1\psi(CH_2–NH)Gly^2$]N/OFQ(1–13)–NH_2 as well as that of Ac-RYYRIK-NH_2 can be manipulated to encompass full and partial agonism along with antagonism in the same cellular environment by changing, as the only variable, NOP receptor density (McDonald et al., 2003). Thus, we propose that Ac-RYYRIK-ol is, similarly to [$Phe^1\psi(CH_2–NH)Gly^2$]N/OFQ(1–13)–NH_2 and Ac-RYYRIK-NH_2, a NOP receptor partial agonist whose estimated pharmacological activity (full, partial agonism or antagonism) strongly depends on the efficiency of the stimulus–response coupling which characterize the different pharmacological preparations (see for a detailed discussion of this topic (Kenakin, 2002)).

In summary our in vitro results confirmed and extended previous findings (Kocsis et al., 2004) demonstrating that Ac-RYYRIK-ol is a potent and selective NOP receptor low efficacy agonist.

This peptide was further characterized in a rather large battery of in vivo assays in mice where it mimicked N/OFQ effects which were previously shown to be sensitive to NOP receptor selective antagonists and/or no longer evident in $NOP^{-/-}$ mice. This applies to the supraspinal pronociceptive (Calo et al., 2002; Nishi et al., 1997; Ozaki et al., 2000), orexigenic (Rizzi et al., 2005) and locomotor inhibiting (Calo et al., 2002; Carra et al., 2005; Nishi et al., 1997; Rizzi et al., 2001) actions as well as to the spinal antinociceptive (Calo et al., 2005; Rizzi et al., 2004) effects. Moreover, the ability of N/OFQ to revert the antidepressant-like effects of the NOP receptor antagonist UFP-101 in the mouse forced swimming (Gavioli et al., 2003) was also shared by Ac-RYYRIK-ol. Thus, in all the in vivo assays we performed, Ac-RYYRIK-ol behaved as a NOP receptor agonist. Again, very similar results were obtained in our and other laboratories by testing the NOP receptor partial agonist [$Phe^1\psi(CH_2–NH)Gly^2$]N/OFQ(1–13)–NH_2 and Ac-RYYRIK-NH_2 in analgesiometric tests after supraspinal (Calo et al., 1998; Candeletti et al., 2000; Wang et al., 1999) or spinal administration (Candeletti et al., 2000; Wang et al., 1999), in locomotor activity (Berger et al., 2000; Rizzi et al., 2001) and food intake (Polidori et al., 2000) studies. These data together with those obtained Ac-RYYRIK-ol in the present study clearly demonstrated that low efficacy agonists behave as full agonists in these in vivo assays. It would be interesting, in future studies, to investigate the actions of Ac-RYYRIK-ol on cardiovascular parameters after i.c.v and i.v. administration; in fact, compounds like [$Phe^1\psi(CH_2–NH)Gly^2$]N/OFQ(1–13)–NH_2 and Ac–RYYRIK–NH_2 have been recently demonstrated to behave as NOP receptor antagonists preventing the hypotension and bradycardia elicited by N/OFQ when injected i.v. while they mimicked N/OFQ hypotensive and bradycardic action after i.c.v. administration (Kapusta et al., 2005a).

In conclusion, Ac-RYYRIK-ol displayed a complex pharmacological profile which is virtually superimposable to that shown by [$Phe^1\psi(CH_2–NH)Gly^2$]N/OFQ(1–13)–NH_2, a well known NOP receptor partial agonist; thus it is likely that Ac-RYYRIK-ol is a low efficacy agonist at NOP receptors. However, the high potency, selectivity of action, metabolic stability and in vivo effectiveness make Ac-RYYRIK-ol a useful pharmacological tool for future studies in the field of N/OFQ and its NOP receptor system.

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