UFP-101 antagonizes the spinal antinociceptive effects of nociceptin/orphanin FQ: Behavioral and electrophysiological studies in mice

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1. Introduction

Nociceptin/orphanin-FQ (N/OFQ) [26,31] is the selective endogenous agonist of the N/OFQ peptide (NOP) receptor [9]. Via selective stimulation of NOP receptor N/OFQ regulates several biological functions both at central (pain transmission, locomotor activity, memory, food intake, response to stress, drug reward) and peripheral (cardiovascular and renal peptides 28 (2007) 663–669 article info

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N/OFQ, nociceptin/orphanin FQ; NOP, nociceptin/orphanin FQ peptide; MOP, mu-opioid peptide; i.t., intrathecal injection; UFP-101, [Nphe'Arg14Lys15]N/OFQ-NH2; EPSC, excitatory postsynaptic currents; IPSC, inhibitory postsynaptic currents; TW, tail withdrawal; NOP+/+, wild type mice; NOP−/−, mice knock out for the NOP receptor gene 0196-9781/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
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functions, motility of the respiratory, gastrointestinal and genitourinary systems) levels [3,27]. Pain transmission is modulated by N/OFQ in a complex manner. In fact, while the supraspinal administration of the peptide produces pronociceptive effects and counteracts opioid and non-opioid induced analgesia, spinally administered N/OFQ evokes antinociceptive effects in a variety of animal models [37]. This latter action may derive from the capability of N/OFQ to inhibit excitatory synaptic transmission in the superficial layers of the dorsal horn [23]. The involvement of NOP receptor antagonist in this N/OFQ action was later demonstrated in receptor knockout studies [1].

Here we used UFP-101 to characterize the receptor involved in the spinal antinociceptive effects of N/OFQ: the NOP antagonist was tested in vitro on excitatory postsynaptic currents (EPSC) recorded in the whole-cell configuration of the patch-clamp technique in laminae I and II of the mouse spinal cord dorsal horn and in vivo after intrathecal (i.t.) administration in the mouse tail withdrawal (TW) assay.

2. Methods

2.1. Animals

Mice were housed in 425 mm x 266 mm x 155 mm cages (Tecniplast, MN, Italy), 8 animals/cage, under standard conditions (22 °C, 55% humidity, 12 h light–dark cycle, light on at 7.00 a.m.) with food (MIL, standard diet, Morini, RE, Italy) and water ad libitum for at least 4 days before experiments began. The animals were handled according to guidelines published in the European Communities Council directives (86/609/EEC) and National Italian Regulation (D.L.116/92). Each animal was used once.

2.2. Tail withdrawal studies

Male Swiss albino mice (5–6-week old, Morini Reggio Emilia, Italy) and CD1/C57-BL6/J/129 (6–7-week old) wild type (NOP+/+) and NOP receptor gene knock out (NOP−/−) mice were used. The latter animals were genotyped by PCR. Details of the generation and breeding of mutant mice have been published previously [14,29]. Intrathecal (i.t.) injection was performed according to the method by Hylden and Wilcox [18]. Briefly, a caudal cutaneous incision (1 cm) was made 2 h before the injection under ether anesthesia. The mouse was held firmly by the pelvic girdle in one hand, while the syringe was held on the other hand at an angle of about 20° above the vertebral column. I.t. injection (5 µl/mouse) was made between L5 and L6. All experiments began at 10.00 a.m. and were performed according to the procedure described by Calo et al. [4]. The animals 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 drug treatment. A cut off time of 20 s was chosen to avoid tissue damage. Tail withdrawal time was determined immediately before and 5, 15, 30 and 60 min after i.t. injection of 5 µl of saline (control), N/OFQ, or endomorphin-1. In some experiments, 10 nmol UFP-101 (i.t.) or naloxone (3 mg/kg s.c. 5 min pretreatment) were tested alone and versus N/OFQ or endomorphin-1.

For each experiment four mice were randomly assigned to each experimental group, and the experiment was repeated at least three times: therefore each experimental point is the mean of the results obtained in ≥12 mice.

2.3. Electrophysiological studies

Ten to 16-day-old mice (C57-BL6/J/129 strain) of either sex were killed by decapitation in deep ether anesthesia. Transverse slices of the lumbar spinal cord (250 µm thick) were prepared as described previously [23]. Slices were completely submerged and continuously superfused with standard external solution containing (in mM) NaCl 125, NaHCO3 26,Na2HPO4 1.25, KCl 2.5, CaCl2 2, MgCl2 1, glucose 10 (pH 7.39) with 95% O2, 5% CO2 at a rate of 1–2 ml/min at room temperature (air conditioned room; 19–21 °C). Whole-cell–patch-clamp recordings were made from neurons of the superficial dorsal horn under visual control visually identified using an infrared gradient contrast equipment [11]. A new slice was used for every recording. Patch pipettes were filled with standard internal solution containing (in mM): K-glutamate 130, KCl 20, MgCl2 2, EGTA 0.05 (acetic acid, ethylenebis(oxyethylenetriolate))tetra-, Na-ATP 3, Na-GTP 0.1 and Na-HEPES 10 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.30). Lidocaine N-ethyl bromide (QX-314) 5 mM was added to the internal solution to block voltage-activated sodium currents and to improve space clamp conditions.

Picrotoxin (50 µM) and strychnine (2 µM) were added to the bathing solution to block inhibitory postsynaptic currents (IPSCs). EPSC were evoked at a frequency of 0.1–0.07 Hz and recorded at ~80 mV at room temperature. Short hyperpolarizing voltage steps to ∼90 mV were applied every minute to monitor input and access resistance. EPSC were elicited by ipsilateral extracellular electrical stimulation (100 µs, 3–10 V) of the dorsal root entry zone using a glass electrode filled with 1 M NaCl. All peptide or drug-containing solutions were applied by bath perfusion. Percent inhibition of EPSC by N/OFQ was determined from the average amplitude of 10 consecutive EPSC evoked immediately before application of the peptides and when a steady state of inhibition was reached, usually about 3 min after peptide application. UFP-101 was applied 5 min before N/OFQ.

2.4. Drugs

The peptides used in this study were prepared and purified as previously described [15]. Naloxone, picrotoxin, strychnine, and QX-314 were from Sigma (Pool, UK). The substances were dissolved freshly on every experimental day.
2.5. Data analysis and terminology

All data are expressed as means ± standard error of the mean (S.E.M.) of n experiments. The pharmacological terminology adopted in this paper is consistent with the IUPHAR recommendations [28]. Raw data from TW experiments were converted to the area under the time (30 min) × withdrawal latency curve and the data expressed as area under the curve (AUC) were used for statistical analysis. The pA2 value of UFP-101 was derived from inhibition experiments using the following equation

\[ pA2 = \log C50 / \left( 2 + \left( [A] / EC50 \right)^{1/n} - 1 \right) \]

where IC50 is the concentration of antagonist that produces 50% inhibition of the agonist response, [A] the concentration of agonist, EC50 the concentration of agonist producing a 50% maximal response, and n is the Hill coefficient of the concentration-response curve to the agonist [19]. The latter two parameters were taken from the previous study by Liebel et al. [23] which was performed under identical experimental conditions.

Data have been analyzed statistically using one-way ANOVA followed by the Dunnett’s test. p-Values less than 0.05 were considered to be significant.

3. Results

3.1. Tail withdrawal studies

In Swiss male mice, the i.t. injection of N/OFQ (0.1 and 1 nmol), endomorphin-1 (3 nmol), UFP-101 (1 and 10 nmol), as well as the systemic administration of naloxone (3 mg/kg) did not produce any effect on animal gross behavior. On the contrary, the i.t. injection of N/OFQ at 10 nmol produced a clear hind limb paralysis and a subsequent decrease in locomotor activity.

In these mice, i.t. injection of N/OFQ in the range of 0.1–10 nmol produced a dose dependent and sustained antinociceptive effect (Fig. 1, left panel). These effects peaked at 5–15 min post injection, and lasted (with the highest dose of N/OFQ) for more than 1 h (AUC saline: 171 ± 19, N/OFQ 0.1 nmol: 191 ± 20; 1 nmol: 277 ± 28; 10 nmol: 367 ± 41, p < 0.05 versus saline according to ANOVA followed by the Dunnett’s test). The antinociceptive effects induced by spinal N/OFQ were compared to those obtained using the selective MOP receptor agonist endomorphin-1. This peptide, at 3 nmol, induced a statistically significant antinociceptive effect, which peaked 5 min after the i.t. injection (Fig. 1, right panel) (AUC saline: 174 ± 16, endomorphin-1: 371 ± 14, p < 0.05 versus saline according to Student’s t-test).

UFP-101 and naloxone were then used to pharmacologically characterize the antinociceptive effects induced by i.t. injection of N/OFQ and endomorphin-1 in Swiss male mice. As shown in Fig. 2 (top panels), naloxone-treated mice showed TW latency values similar to those of saline-injected mice; moreover 3 mg/kg naloxone did not affect the antinociceptive effects induced by 1 nmol of N/OFQ (Fig. 2, top, left panel) (AUC saline: 166 ± 11, N/OFQ: 269 ± 32; naloxone: 167 ± 18, N/OFQ + naloxone: 309 ± 38, p < 0.05 versus saline according to ANOVA followed by the Dunnett’s test), while it almost completely prevented those produced by 3 nmol endomorphin-1 (Fig. 2, top, right panel) (AUC saline: 174 ± 16, endomorphin-1: 317 ± 14; naloxone: 204 ± 33, endomorphin-1 + naloxone: 211 ± 29, p < 0.05 versus saline according to ANOVA followed by the Dunnett’s test). UFP-101-treated mice showed tail withdrawal latency values similar to those of saline-injected mice (Fig. 2 bottom panels). Ten nanomoles of UFP-101 fully prevented the antinociceptive effects induced by 1 nmol N/OFQ (Fig. 2 bottom, left panel) (AUC saline: 189 ± 16, N/OFQ: 345 ± 29; UFP-101: 142 ± 8; N/OFQ + UFP-101: 182 ± 16, p < 0.05 versus saline according to ANOVA followed by the Dunnett’s test) being completely inactive on those induced by 3 nmol endomorphin-1 (Fig. 2 bottom, right panel) (AUC saline: 174 ± 16, endomorphin-1: 317 ± 14; UFP-101: 222 ± 22; endomorphin-1 + UFP-101: 302 ± 26, p < 0.05 versus saline according to ANOVA followed by the Dunnett’s test).

The effects of N/OFQ (10 nmol) and endomorphin-1 (3 nmol) were tested in CD1/C57-BL6/J/129 NOP+/+ and NOP−/− mice. In these mice the i.t. injection of both N/OFQ at 10 nmol

Fig. 1 – Tail withdrawal assay data obtained in Swiss mice. Left panel: dose–response curve to N/OFQ (0.1–10 nmol) injected i.t. (p < 0.05 vs. saline according to ANOVA followed by the Dunnett’s test). Right panel: effect of endomorphin-1 (3 nmol) injected i.t. (p < 0.05 vs. saline according to Student’s t-test). Points represent the means and the vertical bars indicate the S.E.M. of four experiments.
and endomorphin-1 at 3 nmol does not modify the animal gross behavior. As shown in Fig. 3, there were no significant differences in TW latencies between NOP+/+ (4.56 ± 0.27 s) and NOP−/− mice (5.81 ± 0.23 s). In NOP+/+ mice N/OFQ and endomorphin-1 induced a significant antinociceptive effect similar to that observed in Swiss mice (AUC saline: 153 ± 10; endomorphin-1 263 ± 28*, N/OFQ 278 ± 23*, *p < 0.05 versus saline according to ANOVA followed by the Dunnett’s test) while in NOP−/− mice only endomorphin-1 was found to produce a statistically significant antinociceptive action (AUC saline: 199 ± 11; endomorphin-1 283 ± 22*, N/OFQ 236 ± 26, *p < 0.05 versus saline according to ANOVA followed by the Dunnett’s test). It is worthy of mention that both N/OFQ and endomorphin-1 antinociceptive effects were higher and longer lasting in Swiss mice compared to CD1/C57-BL6/J/129 mice.

3.2. Electrophysiological studies

Extracellular application of N/OFQ 1 μM led to a reversible reduction of the EPSC amplitudes by 40 ± 4% (n = 9) without affecting the time course of the currents (Fig. 4, left panel). Steady state levels of inhibition were reached within less than 5 min after drug application. N/OFQ inhibitory effect on EPSC was significantly reversed by UFP-101 in a concentration dependent manner (Fig. 4, right panel). A UFP-101 pA2 value of 6.44 was derived from these experiments. Ten micromolar of UFP 101 did not affect per se EPSC neither in amplitude nor for the time constant, in fact the amplitude of EPSCs in the presence of UFP-101 (10 μM) was 101.2 ± 4% (n = 9) of the control.

4. Discussion

Data obtained in this study confirm that the spinal administration of N/OFQ in mice induces antinociceptive effects, demonstrate the exclusive involvement of NOP receptor in this N/OFQ action, and suggest that activation of NOP receptors blocking glutamatergic transmission in the superficial dorsal horn may represent the mechanism underlying the antinociceptive effect of spinal N/OFQ.

Under the present experimental conditions, spinal administration of N/OFQ in the range of 0.1–10 nmol in mice subjected to the tail withdrawal assay induced clear antinociceptive effects. These results are in line with a large
amount of data obtained in mice and rats using different analgesiometric tests (see for reviews [27,37]). In the present study N/OFQ potency (0.1 nmol threshold dose, 1 nmol induces statistically significant effects, 10 nmol represents the maximal dose) is very similar to the one found by our group in a previous study investigating the supraspinal pronociceptive action of the peptide [4]. The only difference is in the peptide duration of action: the antinociceptive effects elicited by spinal administration of N/OFQ are longer lasting (>60 min) compared to the pronociceptive ones induced by supraspinal administration of N/OFQ which do last no longer than 30 min after the injection. Since it has been demonstrated that peptidase activities regulate N/OFQ actions [30], we may speculate that the different duration of action of N/OFQ at spinal and supraspinal sites can be due to a higher expression/activity of peptidases in the brain compared to the spinal cord.

Endomorphin-1 given i.t. induced antinociceptive effects similar to those evoked by N/OFQ. However, the MOP selective receptor agonist showed the same pattern of action (i.e. antinociceptive effects) both when administered spinally and supraspinally [36]. Since the cellular actions of MOP and NOP receptors (i.e. inhibition of cAMP levels, activation of K+ and inhibition of Ca2+ currents [17]) are virtually identical and their effects on pain transmission are similar or opposite depending on the site of administration, it is possible to speculate a different cellular localization of MOP and NOP receptors in brain but overlapping expression in spinal areas implied in the processing of nociceptive stimuli. Electrophysiological findings obtained in different laboratories and reviewed in [37] support this interpretation.

Pharmacological and knockout data obtained in the present study clearly demonstrated that the spinal analgesic
effect of N/OFQ is exclusively due to NOP receptor activation. In fact, these effects of N/OFQ were (i) naloxone resistant, (ii) sensitive to the antagonist action of UFP-101, and (iii) no longer evident in NOP\(^{-/-}\) mice. On the other hand, endomorphin-1 actions were sensitive to naloxone, not affected by UFP-101 and similar in NOP\(^{+/+}\) and NOP\(^{-/-}\) mice, thus suggesting the involvement of classical opioid receptors in its action.

UFP-101 completely prevented N/OFQ effects, but not those induced by endomorphin-1, at an antagonist/agonist dose ratio, i.e. 10/1, similar to that used by different groups to counteract several actions of N/OFQ including supraspinal pronociceptive effects [6], inhibition of locomotor activity [6,22] and rotarod performance [25], anxiolytic like effects [33], stimulation of food intake [12], bradycardia and hypotension [16], diuresis [21], and inhibition of gut motor and secretory functions [2]. Thus, the present data confirm the selectivity of action of UFP-101 and its in vivo antagonist potency and, together with those from literature, the usefulness of this NOP ligand for in vivo pharmacological and pathophysiological studies.

The present pharmacological and knockout findings are in line with other evidence indicating that N/OFQ spinal antinociceptive effect is solely due to NOP receptor activation. In fact, the antinociceptive action of N/OFQ given i.t. is prevented by selective NOP antagonists such as the non-peptide J-113397 [20] or the peptide [Nphe\(^{1}\)N/OFQ(1-13)NH\(_2\)]\(^{8,13,34}\).

The mechanisms underlying N/OFQ spinal antinociceptive action of are not completely known. However, it is worthy of mention that in the spinal cord dorsal horn, N/OFQ selectively inhibits excitatory neurons and reduces synaptic release of glutamate [23]. This action of N/OFQ is absent in NOP\(^{-/-}\) mice [1], resistant to naloxone [23] while sensitive to the NOP selective antagonist J-113397 [24]. In addition, the present results demonstrated that the inhibitory effect of N/OFQ on spinal glutamatergic transmission is sensitive to the antagonist action of UFP-101, which displayed a pA\(_2\) value of 6.44. This value of UFP-101 antagonist potency is rather low compared to range of values (7.0–7.5) obtained in several different preparations investigated with different techniques (see data reviewed in [5]). However, it must be noticed that in this particular preparation also the agonist potency of N/OFQ (pEC\(_{50}\) approximately 5.5 [1] and 6.3 [23] in mouse and rat tissues, respectively) is lower than that recorded in several other preparations (range 7.2–8.6 [5]). The unusual low potency of NOP agonists and antagonists evaluated in spinal cord slices might derive from a particularly low accessibility to ligands of NOP receptor expressed in this preparation.

Collectively, the pharmacological features of N/OFQ inhibition of EPSCs in spinal cord slices parallel those of N/OFQ induced spinal antinociception (see above) strongly suggesting that presynaptic inhibition of glutamatergic excitatory transmission in the dorsal horn of the spinal cord represents the neurochemical mechanism of the antinociceptive response to spinal N/OFQ.

Spinal application of UFP-101 at doses effective in blocking the action of exogenous N/OFQ does not modify per se TW latencies, indicating that, under the present experimental conditions, pain transmission is not tonically controlled by the endogenous spinal N/OFQ–NOP receptor system. This is corroborated by the fact that TW latencies are similar in NOP\(^{+/+}\) and NOP\(^{-/-}\) mice [29] and present data. However, several evidences indicate that spinal N/OFQergic signaling could be activated and modulate pain transmission in response to tonic/prolonged nociceptive stimuli. In fact, in the formalin test spinal administration of UFP-101 as well as systemic administration of J-113397 produces pronociceptive effects [32,35] and NOP\(^{-/-}\) mice (as well as mice knock out for the N/OFQ peptide precursor) display a robust pronociceptive phenotype [10,32]. Collectively these findings suggest that activation of the endogenous N/OFQ–NOP receptor system at spinal level becomes operative only during tonic nociceptive stimulation.

Collectively data presented in this study confirm and extend previous findings demonstrating that spinally delivered N/OFQ evokes robust antinociceptive effects due to NOP receptor activation, which (most likely) causes inhibition of glutamatergic primary afferent fibers. Antinociceptive effects evoked by spinal administration of N/OFQ were recently reported in primates [20] thus making spinal NOP receptors an attractive target for the development of innovative spinal analgesics for human use.

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