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C-Terminal glycine is crucial for hyperalgesic activity of nociceptin/orphanin FQ-(1–6)

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Abstract

A C-terminal analog of the hexapeptide orphanin FQ/nociceptin-(1–6), [Ala^6]-orphanin FQ/nociceptin-(1–6), and a pentapeptide orphanin FQ/nociceptin-(1–5) were tested in vivo for their analgesic/hyperalgesic activity in the hot-plate test with rats. Replacement of the C-terminal glycine by L-alanine (Phe-Gly-Gly-Phe-Thr-Ala) in orphanin FQ/nociceptin-(1–6) abolished the hyperalgesic potency of native orphanin FQ/nociceptin-(1–6) (Phe-Gly-Gly-Phe-Thr-Gly), but analgesic activity was retained and was diminished by naloxone. Removal of the C-terminal amino acid (glycine or alanine) from orphanin FQ/nociceptin-(1–6) caused a significant loss of analgesic activity. It is anticipated that glycine plays a crucial role in the biphasic activity of orphanin FQ/nociceptin-(1–6). This may suggest the existence of a mechanism for terminating the biological action of orphanin FQ/nociceptin.

Keywords: Orphanin FQ/nociceptin; Metabolism; Glycine; Opioid receptor; Pain

1. Introduction

Orphanin FQ (Reinscheid et al., 1995) or nociceptin (Meunier et al., 1995) is a newly discovered natural agonist of the orphan opioid receptor-like-1 (ORL1) receptor. Orphanin FQ/nociceptin is a 17-amino acid long peptide (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) and has homology with the opioid family of peptides, particularly dynorphin A (Julius 1995; Houtani et al., 1996). Its sequence lacks the N-terminal L-tyrosine essential for binding to the μ-, δ- and κ-opioid receptors (Reinscheid et al., 1995; Chavkin and Goldstein, 1981). Because of this, orphanin FQ/nociceptin has been classified as belonging to a separate peptidergic system. Orphanin FQ/nociceptin, similar to opioid peptides, is present in the central nervous system (CNS) (Saito et al., 1996). Orphanin FQ/nociceptin and opioid peptides show an overlapping distribution but not co-localization, e.g. in pain-modulatory brain regions (Schulz et al., 1996).

The ORL1 receptors are widely distributed in the rat CNS, with high levels found in the cortex, olfactory nucleus, amygdala, claustrum and endopiriform nucleus (Anton et al., 1996). Despite the close similarity to opioid receptors (approximately 65% sequential homology with μ-, δ- and κ-opioid receptors), ORL1 does not bind, with high affinity, any of the previously identified opioid peptides or other ligands. Moreover, orphanin FQ/nociceptin is unable to activate opioid receptors (Reinscheid et al., 1995).

Orphanin FQ/nociceptin exhibits actions, which differ from those of traditional opioids in pharmacological studies. It has been reported that intracerebroventricular (i.c.v.) administration of orphanin FQ/nociceptin induces hyperalgesia (Reinscheid et al., 1995; Meunier et al., 1995; Hara et al., 1997) or an anti-opioid effect (Mogil et al., 1996).

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However, intrathecal (i.t.) injection of this peptide produces an antinociceptive effect at high doses (Xu et al., 1996), and allodynia or hyperalgesia at low doses (Okuda-Ashitaka et al., 1996). Several reports indicate that the hyperalgesic effect of i.c.v. injected orphanin FQ/nociceptin is followed by analgesia (Rossi et al., 1997; King et al., 1997). Antisense mapping studies suggested that the hyperalgesic and analgesic actions of orphanin FQ/nociceptin are mediated through different receptors (Rossi et al., 1998). These data strongly suggest that orphanin FQ/nociceptin is involved in pain transmission and/or in the pain regulation system(s), but the details of these processes remain unclear.

The complex pharmacology of orphanin FQ/nociceptin suggests that at least some of the observed effects may be exerted by shorter orphanin FQ/nociceptin fragments. Recently, Montiel et al. (1997) have shown that the N-terminal fragment orphanin FQ/nociceptin-(1–7) is a major nociceptin metabolite in mouse brain cortical slices. Our previous studies have shown that orphanin FQ/nociceptin-(1–11) and orphanin FQ/nociceptin-(1–6) are released by a proteinase present in the rat spinal cord (Smoluch et al., 1999) and confirmed their biological activity in vivo after i.c.v. injection (Suder et al., 1999). Their action exhibited a biphasic effect, opposite to that produced by native orphanin FQ/nociceptin, causing antinociception up to 10 min after injection, followed by hyperalgesia (Suder et al., 1999). The biological activity of the hexapeptide was more pronounced than that of orphanin FQ/nociceptin-(1–11). It is worth noting that orphanin FQ/nociceptin-(1–6) contains a C-terminal glycine residue, which might be important for triggering its hyperalgesic effect. The aim of the present study was to test to which extent replacement of the C-terminal amino acid of orphanin FQ/nociceptin-(1–6) or its removal influences the biological activity of this peptide.

2. Materials and methods

2.1. Animals

In vivo experiments were performed according to the procedure described previously (Suder et al., 1999). Male Wistar rats (180–250 g) were housed in groups of five, with standard food and water ad libitum and maintained under a 12-h light/dark cycle. After 1 week of adaptation and handling, the animals were prepared for intracerebroventricular injections (i.c.v.) 2 days before the experiment. Surgery was done under light anesthesia with 7.2% chloralum hydratum (Cefarm, Lublin, Poland). The coordinates for i.c.v. injections were measured from the bregma, according to the atlas of König and Klippel (1963) and were as follows: 1.5 mm lateral, 1.0 mm caudal, 3.5 mm ventral from the surface of the skull into the right lateral ventricle. On the day of experiment, the rats received a single injection of either saline (controls), or 10 and 20 μg [Ala^6]-orphanin FQ/nociceptin-(1–6) or orphanin FQ/nociceptin-(1–5) in a volume of 5 μl.

2.2. Behavioral studies

Nociceptive responses were assessed in a hot-plate assay. In this test, rats (7–15 rats/group) were placed on a warm surface (Porfex, Bialystok, Poland) heated to 56°C (Maves and Gebhart, 1992; Yaksh et al., 1985) and the latency to avoidance or withdrawal behavior (foot lifting or jumping) to the thermal stimulus was determined. Thermal responses of rats were measured 2, 10, 20, 30, 40, 50 and 60 min after i.c.v. injections of the peptides. The maximal time spent by the animals in the apparatus did not exceed 20 s to avoid tissue damage. All experiments were performed in agreement with ethical regulations and were approved by the local Ethics Committee.

2.3. Peptides and chemicals

Orphanin FQ/nociceptin fragments were synthesized by Fmoc (9-fluorenylmethoxycarbonyl) chemistry on a solid-phase support, and their sequences are listed in Fig. 1. The peptides were purified on a reversed-phase HPLC (high-performance liquid chromatography) column. Their purity was greater than 95% and was tested by electrospray ionization mass spectrometry (Smoluch et al., 1999; Suder et al., 1999).

Naloxone hydrochloride (Sigma-Aldrich) was dissolved in saline and administered intraperitoneally (i.p.) at a dose of 5 mg/kg, 15 min before peptide injection. Peptides were dissolved in 0.9% NaCl.

Other reagents and solvents were of analytical grade (or HPLC grade) and were purchased from various commercial sources.

2.4. Statistical analysis

Statistical evaluation was performed using the one-way analysis of variance (ANOVA), followed by a Tukey–Kramer multiple comparisons test. A value of $P < 0.05$ was considered as being statistically significant.

Orphanin FQ/nociceptin-(1-6)	Phe-Gly-Gly-Phe-Thr-Gly
[Ala^6]Orphanin FQ/nociceptin-(1-6)	Phe-Gly-Gly-Phe-Thr-Ala
Orphanin FQ/nociceptin-(1-5)	Phe-Gly-Gly-Phe-Thr

Fig. 1. Sequences of the orphanin FQ/nociceptin (OFQ/N)-derived peptides used in this study.

3. Results

[Ala^6]-Orphanin FQ/nociceptin-(1–6) induced analgesia at 10 min after administration at a dose of 20 μg ($P < 0.01$, Fig. 2, upper panel), and this effect was observed as a prolongation of the withdrawal reaction of rats (foot lifting or jumping) in the hot-plate test. This analgesic effect was reversed by injection of an opioid receptor antagonist, naloxone ($P < 0.01$) at a dose 5 mg/kg. For data comparison, the effect of the unmodified hexapeptide orphanin FQ/nociceptin-(1–6) is also shown in Fig. 2 (bottom panel). Administration of the pentapeptide lacking the C-terminal glycine orphanin FQ/nociceptin-(1–5) at the same doses (10 and 20 μg/rat, i.c.v.) did not produce any biological activity in the hot-plate test (Fig. 3).

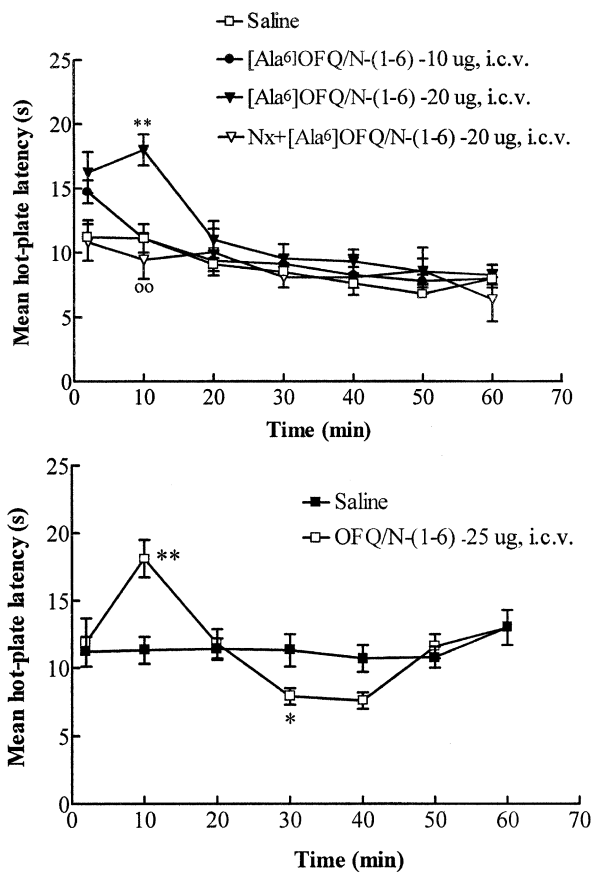

Fig. 2. The effect of the i.c.v. administration of [Ala^6]-orphanin FQ/nociceptin-(1–6) (upper panel) (10 and 20 μg/rat; $N = 10$ and 15, respectively) in the hot-plate test with rats. Injection of [Ala^6]-orphanin FQ/nociceptin-(1–6) at the dose of 20 μg i.c.v. produced an analgesic effect during the first 10 min of observation ($^{**}P < 0.01$; $N = 7$), and this effect was abolished by an opioid antagonist—naloxone ($^{oo}P < 0.01$; $N = 9$). For comparison, the lower panel shows the effect of the native fragment of orphanin FQ/nociceptin-(1–6) in the hot-plate test with rats ($N = 10$). This fragment is formed by enzymatic degradation in the spinal cord. Orphanin FQ/nociceptin-(1–6) at the dose of 25 μg/rat i.c.v. produced analgesic effect during the first 20 min after injection, with highest activity at 10 min of observation ($^{**}P < 0.01$; $N = 14$), followed by hyperalgesia at 30 min ($^{*}P < 0.05$). Data represent results that are representative for all experiments, which were performed independently on various days (three times). Abbreviations: OFQ/N—orphanin FQ/nociceptin.

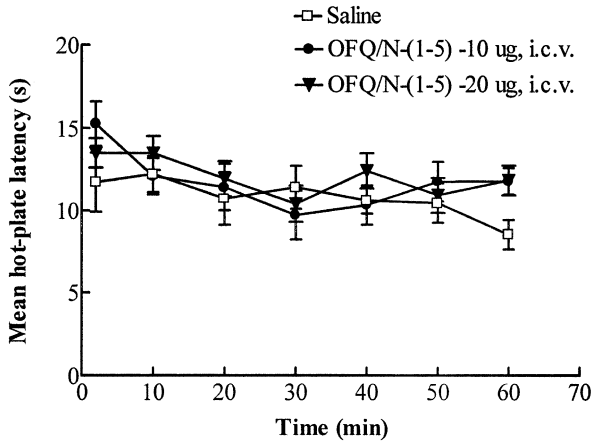

Fig. 3. The influence of orphanin FQ/nociceptin-(1–5) at doses of 10 and 20 μg/rat i.c.v. on rats' behavior in the analgesic hot-plate test ($N = 8$ and 12, respectively). Experimental details are described in the legend of Fig. 2 and in Section 2. Abbreviations: OFQ/N—orphanin FQ/nociceptin.

4. Discussion

As shown in our previously published paper (Suder et al., 1999), orphanin FQ/nociceptin-(1–6) injected at a dose of 25 μg i.c.v. resulted in a biphasic effect (Fig. 2) in the hot-plate test with rats. Hexapeptide produced pronounced analgesia with a maximal effect appearing at 10 min after injection, followed by hyperalgesia, which occurred 30 min after i.c.v. administration of the peptide. The analgesia was reversed by administration of the opioid antagonist naloxone, and hyperalgesia was inhibited by noncompetitive *N*-methyl-D-aspartate (NMDA) and NMDA/glycine site antagonists (Suder et al., 1999). Our present data indicate that modification of the C-terminal end of orphanin FQ/nociceptin-(1–6) by replacement of Gly by Ala results in only an analgesic effect without any hyperalgesic reaction in the hot-plate test with rats. Removal of the C-terminal amino acid (Gly or Ala) resulted in a loss of biological activity in this test (Fig. 3).

Orphanin FQ/nociceptin derives from the larger precursor pre-pronociceptin (Reinscheid et al., 1995; Meunier et al., 1995; Houtani et al., 1996). In the spinal cord, orphanin FQ/nociceptin is consecutively metabolized by a neutral proteinase (Smoluch et al., 1999; Suder et al., 1999) to orphanin FQ/nociceptin-(1–11), followed by its further cleavage to the final product orphanin FQ/nociceptin-(1–6). Another metabolic pathway was described by Montiel et al. (1997) in which the concerted action of aminopeptidase N and endopeptidase 24.15 was involved. Metabolic fragments of orphanin FQ/nociceptin induce behavioral responses in analgesic tests (Rossi et al., 1997;

King et al., 1997; Suder et al., 1999). The effect of these events (analgesia followed by hyperalgesia) is opposite to that caused by supraspinal injection of native orphanin FQ/nociceptin (hyperalgesia followed by an analgesia). Therefore, the ratio between the native peptide and its fragments may be crucial in pain control (Sakurada et al., 1999) and also in other effects of orphanin FQ/nociceptin (Sakurada et al., 2000). In this respect, an enzyme that converts orphanin FQ/nociceptin to shorter products might play a key role in these processes.

Replacement of the glycine by L-alanine preserved the analgesic action of orphanin FQ/nociceptin-(1–6) hexapeptide but completely terminated its hyperalgesic properties. Moreover, removal of the C-terminal amino acid, which resulted in the formation of the pentapeptide orphanin FQ/nociceptin-(1–5), resulted in a complete loss of pharmacological activity, as tested in the hot-plate test. In vivo, the C-terminal amino acids can be removed by the action of carboxypeptidases (Fricker and Leiter, 1999). Another mechanism of peptide alteration is based on the modification of the C-terminal glycine residue by a peptidyl-glycine-α-amidating monooxygenase (Lapalu et al., 1997), which converts glycine into its amide. During our experiments (limited proteolysis), we observed the formation of small amounts of the pentapeptide (data not shown), suggesting that the glycine residue is, indeed, removed during orphanin FQ/nociceptin metabolism. There was no mass shift of 1 amu in the signal obtained in the mass spectrum and corresponding to orphanin FQ/nociceptin-(1–6), which indicates that amidation of the C-terminus did not take place under our experimental conditions.

It is rather unlikely that we overlooked hyperalgesia just for one particular peptide. The tests we and others used have been routinely used in our laboratory for many years. To avoid any misinterpretation, we repeated the experiments several times, with various doses and performed dose-dependent studies. Therefore, we can exclude the possibility that only [Ala^6]-orphanin FQ/nociceptin-(1–6) can produce an effect other than the one described by us under given experimental conditions.

Using the chimeric peptide approach, Lapalu et al. (1997) indicated that the Thr^5-Gly^6 sequence is not important for ORL1 receptor binding selectivity, and that several hybrid peptides (nociceptin/dynorphin hybrid heptadecapeptides) containing the Phe-Gly-Gly-Phe sequence are completely inactive towards the ORL1 receptor (Mathis et al., 1999). Moreover, application of the orphanin FQ/nociceptin-(1–11) analog- [^{125}I]-[Tyr^{10}]-orphanin FQ/nociceptin-(1–11) (Mathis et al., 1999) identified a high-affinity binding site with a selective profile distinct from that of the ORL1 receptor and all other "classical" opioid receptors. This site had a very high affinity for orphanin FQ/nociceptin and related peptides, and several opioids displayed a moderate affinity for this site. Taking into account the above facts, it is not unlikely that the analgesic effect of orphanin FQ/nociceptin-(1–6) or [Ala^6]-orphanin FQ/nociceptin-(1–6) observed in our studies, although abolished by naloxone, may be a result of an interaction of these peptides with a subtype of the ORL1 receptor.

It should be noted that, in the present work, we describe an orphanin FQ/nociceptin fragment (1–6) which does not bind to the ORL-1 receptor. Similar studies have been performed by several groups (e.g. Civelli's and Meunier's), thus, strongly indicating that shorter fragments lose their ability to bind to the ORL1 receptor. Therefore, the use of ORL1 antagonists for studies on the intact orphanin FQ/nociceptin sequence, in particular for further investigation of its effect on the morphine withdrawal syndrome (Kotlińska et al., 2000) will be of our interest in the nearest future.

Our results indicate that the glycine residue plays a crucial role for the biphasic activity of orphanin FQ/nociceptin-(1–6) in the hot-plate test, and that the removal of the amino acid from this peptide fragment causes a loss of its biological activity. This observation may suggest the existence of a mechanism for terminating the biological action of orphanin FQ/nociceptin in the CNS.

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