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Synthesis and biological activity of nociceptin/orphanin FQ(1–13)NH_2 analogues modified in 9 and/or 13 position

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Abstract—The purpose of the present study was the synthesis and the biological screening of new analogues of N/OFQ(1–13)NH_2, the minimal sequence maintaining the same activity as the natural peptide nociceptin. In order to investigate the role of Lys, we substituted Lys at positions 9 and/or 13 by Orn, Dab (diaminobutanoic acid) or Dap (diaminopropanoic acid). The new N/OFQ(1–13)NH_2 analogues exerted strong and naloxone-resistant inhibition of electrically evoked contractions of rat vas deferens. Lys replacement with Orn maintained or even enhanced the inhibitory activity, while replacements with Dab and Dap decreased inhibitory activity.

Nociceptin, also known as orphanin FQ (N/OFQ), is a neuropeptide, structurally related to opioid peptides (especially dynorphin A), which does not interact with classical opioid receptors. The heptadecapeptide N/OFQ (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) selectively activates its own receptor NOP, previously named opioid receptor-like 1 (ORL_1) or OP_4, a novel member of the opioid receptor family.[1,2] It has been shown that the NOP-receptor and N/OFQ are expressed not only in the central nervous system (CNS) but also in peripheral tissues, suggesting that this novel peptide–receptor system might modulate both central and peripheral biological functions.[3–6] At central sites, N/OFQ modulates pain, anxiety, feeding, locomotion, learning and memory. In periphery, it affects cardiovascular, gastrointestinal, urogenital and respiratory functions. Guerrini et al.[7] proposed that the N-terminal tridecapeptide sequence of the nociceptin molecule suffices for its full biological activities. It has been reported that Arg,[8,12] Lys[9,13] seem to be crucial for receptor occupation, most probably interacting with the acidic amino acids present in the second extracellular loop of the NOP-receptor.[8,9] Structure–activity relationship (SAR) studies demonstrated that the N/OFQ sequence can be divided into a N-terminal tetrapeptide 'message' crucial for receptor activation and a C-terminal 'address' important for receptor binding.[10]

Based on the 'message/address' concept we aimed to investigate the importance of the Lys and distance of the side-chain amino group from the peptide backbone on the biological activity. We substituted Lys at positions 9 and/or 13 by its own structural analogues Orn, diaminobutanoic acid (Dab) or diaminopropanoic acid (Dap) in the N/OFQ(1–13)NH_2 template. Lysine was chosen on the assumption that cationic residues (as Arg,[8,12] Lys[9,13]) appear to play a functional role for receptor binding.[7,10]

The solid-phase peptide synthesis by Fmoc (9-fluorenylmethoxycarbonyl) chemistry was used to obtain several structural analogues of N/OFQ(1–13)NH_2 by replacing Lys at positions 9 and/or 13 with Orn, Dab or Dap.[11]

The crude peptides were purified on a reversed-phase high-performance liquid chromatography (HPLC) and the molecular weights determined using electrospray ionization mass-spectrometry.[12]

The new peptides have the following sequences:

H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-X^9-Ser-Ala-Arg-X^{13}-NH_2

Keywords: Opioid peptides; Nociceptin/orphanin FQ; NOP; Nociceptin analogues; SPPS; Rat vas deferens.
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$X^{9,13}$ = Lys (**1**); X^{9} = Lys, X^{13} = Orn (**2**); X^{9} = Orn, X^{13} = Lys (**3**); $X^{9,13}$ = Orn (**4**); $X^{9,13}$ = Dab (**5**); $X^{9,13}$ = Dap (**6**).

The side chain of each consecutive compound is one methylene group shorter.

$$H_2N{-}CH(R){-}COOH$$

Lys, R = $(CH_2)_4NH_2$; Orn, R = $(CH_2)_3NH_2$; Dab, R = $(CH_2)_2NH_2$; Dap, R = $(CH_2)NH_2$

The biological activities of the parent compound and its analogues were tested in vitro on electrically stimulated rat vas deferens.[13]

Cumulatively administered in concentrations of 1×10^{-8} to 1×10^{-5} M, none of the newly synthesized N/OFQ-analogues markedly affected the tone of the isolated smooth muscles. The EC_{50} values and the E_{max} of the newly synthesized peptides on the LFES-evoked smooth-muscle contractions are presented in Table 1.

In order to elucidate the receptors involved in the smooth-muscle inhibitory effects of the new peptides, experiments were carried out with 1×10^{-6} M NAL that blocks the classical opioid—but not the NOP-receptors. In this experimental series, no significant changes in the inhibition of the smooth-muscle contractions by the tested peptides were registered (Figs. 1–4 and Table 1).

As shown on Table 1, the replacement of Lys with Orn at position 9 enhanced the inhibitory potency and the maximum effect of N/OFQ(1–13)NH_2. The same substitution at position 13 exerted an opposite effect on the biological activity of N/OFQ(1–13)NH_2. Therefore, it might be suggested that the side-chain reduction with one CH_2 group at position 9 resulted in increased inhibitory activity, whilst the same shortening of the side chain at position 13 led to a pronounced decrease in the inhibitory potency of the compound. Simultaneous substitution at positions 9 and 13 reduced the inhibitory effects of the parent compound on the LFES-induced contractions of rat vas deferens.

The present results revealed that the new N/OFQ-analogues might exert inhibitory effects on smooth-muscle contractions, and that this action probably did not involve classical opioid receptors.

Our results showed that the opioid antagonist naloxone did not change the strong inhibitory action of the newly synthesized N/OFQ(1–13)NH_2-analogues on LFES-evoked contractions. The data corroborate with the results obtained on the same tissue by Rizzi et al.[14] It might be suggested, therefore, that classical opioid receptors were not involved in the inhibitory activity of the new N/OFQ-analogues. Most probably, their effects are due to interactions with NOP-receptors; since the changes in the N/OFQ(1–13)NH_2 molecule have been made in its 'address' part, the observed differences in the biological activity of the new compounds might be due to variations in their affinity towards NOP-receptors. Another possibility is that the peptides tested

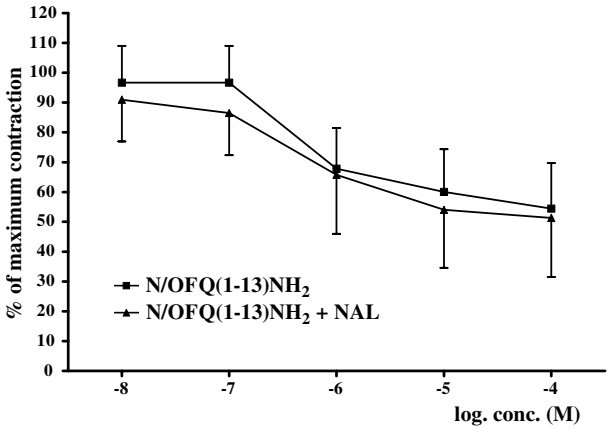

Figure 1. Rat vas deferens. Concentration–response curve of N/OFQ(1–13)NH_2 before and after naloxone, on the contractions induced by LFES. The data are means ± SEM of seven experiments.

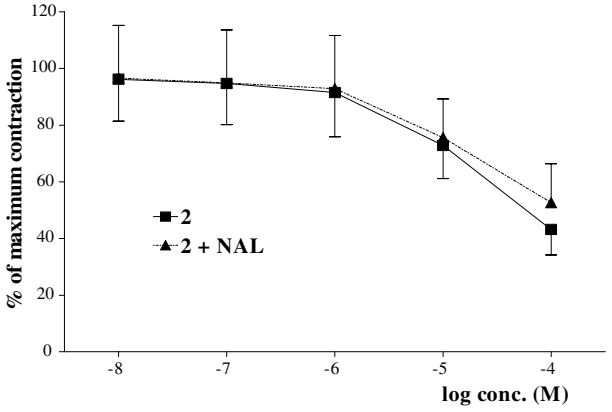

Figure 2. Rat vas deferens. Concentration–response curve of [Orn^{13}] N/OFQ(1–13)NH_2 before and after naloxone, on the contractions induced by LFES. The data are means ± SEM of nine experiments.

Table 1. Effects and potency of the newly synthesized peptides on LFES-evoked contractions of rat vas deferens

Compound	Peptides tested	Peptide alone			NAL + peptide		
		pEC_{50}	E_{max} (%)	Relative potency	pEC_{50}	E_{max} (%)	Relative potency
1	N/OFQ(1–13)NH_2	6.24 ± 0.30	−46 ± 15	1.00	6.40 ± 0.36	−49 ± 20	1.00
2	[Orn^{13}]N/OFQ(1–13)NH_2	4.95 ± 0.14	−57 ± 9	0.05	4.96 ± 0.19	−49 ± 8	0.04
3	[Orn^{9}]N/OFQ(1–13)NH_2	6.63 ± 0.20	−71 ± 7	2.45	6.67 ± 0.12	−77 ± 4	1.56
4	[$Orn^{9,13}$]N/OFQ(1–13)NH_2	6.39 ± 0.30	−56 ± 13	1.41	6.01 ± 0.27	−70 ± 9	2.45
5	[$Dab^{9,13}$]N/OFQ(1–13)NH_2	5.21 ± 0.19	−60 ± 12	0.09	5.03 ± 0.20	−33 ± 21	0.04
6	[$Dap^{9,13}$]N/OFQ(1–13)NH_2	5.63 ± 0.15	−54 ± 13	0.25	5.21 ± 0.17	−46 ± 12	0.06

Data represent mean values ± SEM of at least six separate experiments.

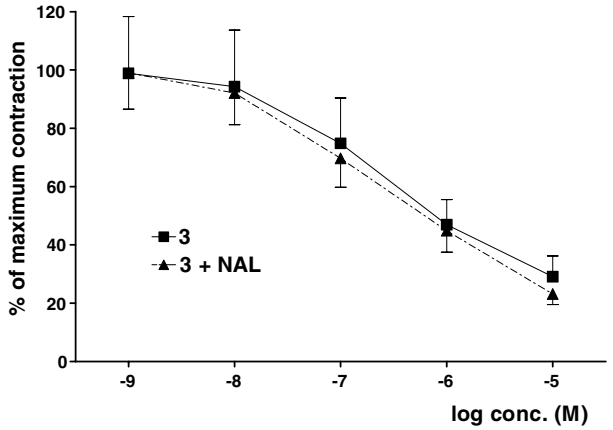

Figure 3. Rat vas deferens. Concentration–response curve of [Orn^9]N/OFQ(1–13)NH_2 before and after naloxone, on the contractions induced by LFES. The data are means ± SEM of 11 experiments.

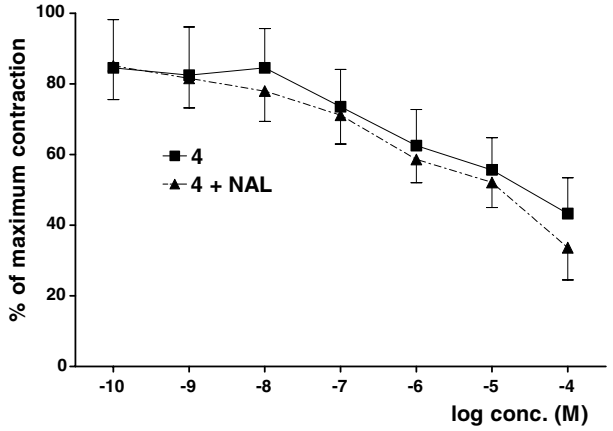

Figure 4. Rat vas deferens. Concentration–response curve of [Orn^{9,13}]N/OFQ(1–13)NH_2 before and after naloxone, on the contractions induced by LFES. The data are means ± SEM of nine experiments.

act via mechanisms independent of the N/OFQ-receptor, as well as of the μ-, δ- and κ-opioid receptors.[15]

The results obtained suggested that replacement of Lys by Orn at position 9 statistically significantly enhanced the biological activity, compared to the other newly synthesized nociceptin analogues; substitution at position 13 exerted an opposite effect, decreasing the biological activity of N/OFQ(1–13)NH_2. These data revealed the importance of the side-chain length of the amino acid at positions 9 and 13 for the biological activity of nociceptin derivatives.

Acknowledgments

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11. *Synthesis.* The solid-phase peptide synthesis by Fmoc (9-fluorenylmethoxycarbonyl) chemistry was used to obtain nociceptin/orphanin FQ fragments **1**–**6**. Rink-amide resin was used as a solid-phase carrier, and 2-(1-OH-benzotriazole-1-yl)1,1,3,3-tetramethyl-carbamide tetrafluoroborat (TBTU) was used as a coupling reagent. The 3-functional amino acids were embedded as follows: Arg—as N^{α}-Fmoc-Arg(Pbf)-OH, Lys—as N^{α}-Fmoc-Lys(Boc)-OH, Orn—as N^{α}-Fmoc-Orn(Boc)-OH, Dab—as N^{α}-Fmoc-Dab(Boc)-OH, Dap—as N^{α}-Fmoc-Dap(Boc)-OH, Ser—as N^{α}-Fmoc-Ser(^{t}Bu)-OH and Thr—as N^{α}-Fmoc-Thr(^{t}Bu)-OH. All coupling reactions were performed, using for amino acid/TBTU/HOBt/DIEA/resin a molar ratio of 3/2.9/3/6/1. The Fmoc-group was deprotected by a 20% piperidine solution in dimethylformamide. The coupling and deprotection reactions were checked by the Kaiser test. The cleavage of the synthesized peptide from the resin was done, using a mixture of 95 % trifluoroacetic acid (TFA), 2.5 % triisopropylsilan (TIS) and 2.5 % water. The protected amino acids were purchased from IrisBiotech (Germany). All other reagents and solvents were analytical or HPLC grade and were bought from Merck (Germany).
12. The crude peptides were purified on a reversed-phase high-performance liquid chromatography (HPLC) C_{18} column, using gradient elution with the following solvents: A—H_2O/0.1% TFA and B—CH_3CN/0.1% TFA. The peptide purity was checked by electrospray ionization mass-spectrometry.The analytical data for the synthetic peptides prepared were as follows: compound **2** t_R 7.89 min, >98% pure, 1368.6 calculated (MH^+), 1368.3 observed (MH^+); compound **3** t_R 7.91 min, >99% pure, 1368.6 calculated (MH^+), 1368.5 observed (MH^+); compound **4** t_R 6.80 min, >99% pure, 1354.4 calculated (MH^+), 1354.6 observed (MH^+); compound **5** t_R 7.22 min, >97% pure, 1326.6 calculated (MH^+), 1326.3 observed (MH^+); **6** t_R 6.98 min, >98% pure, 1299.4 calculated (MH^+), 1299.5 observed (MH^+).
13. *Biological assay.* The biological activity of the newly synthesized N/OFQ analogues was tested in vitro on vas deferens smooth muscles, isolated from male Wistar

rats, weighing 200–250 g. After killing the animals, both vasa deferentia were carefully cleaned from the surrounding connective tissues and blood vessels. Prostatic segments, 12–15 mm long, were fixed in 4 ml organ baths, being connected to electronic transducers. Before the experiment, the smooth muscles were pre-loaded with 1 g, and left for a 30 min adaptation at 32.5 °C in Krebs solution, aerated with 95% O_2 and 5% CO_2. Smooth-muscle contractions were evoked by low-frequency electrical stimulations (LFES) with parameters: 0.05 Hz frequency, 1 ms pulse duration, sub-maximal voltage. The smooth-muscle tone and the LFES-induced contractions were isometrically registered. The tested compounds (1×10^{-8} to 1×10^{-4} M) were cumulatively applied into the organ bath. In some experiments, 1×10^{-6} M naloxone (NAL) was administered 10 min before the examined substance. The E_{max} values represented the maximum effect that the peptide could elicit in the smooth-muscle preparation used. The potencies (pEC_{50}) of the newly synthesized peptides were measured as the negative logarithm of the molar concentration of the compound that induces 50% of its maximum effect. The relative potencies were calculated, assuming the potency of N/OFQ(1–13)NH_2 as a unit. All experiments on isolated smooth muscles have been performed according to the rules of the Ethics Committee of the Institute of Physiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

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