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ORIGINAL ARTICLE

Synthesis and biological activity of new series of N-modified analogues of the N/OFQ(1–13)NH_2 with aminophosphonate moiety

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Abstract New series of N-modified analogues of the N/OFQ(1–13)NH_2 with aminophosphonate moiety have been synthesized and investigated for biological activity. These peptides were prepared by solid-phase peptide synthesis—Fmoc-strategy. The N/OFQ(1–13)NH_2 analogues were tested for agonistic activity in vitro on electrically stimulated rat vas deferens smooth-muscle preparations isolated from Wistar albino rats. Our study has shown that the selectivity of the peptides containing 1-[(methoxyphosphono)methylamino]cycloalkanecarboxylic acids to the N-side of Phe is not changed—they remain selective agonists of NOP receptors. The derivative with the largest ring (**NOC-6**) demonstrated efficacy similar to that of N/OFQ(1–13)NH_2, but in a 10-fold higher concentration. The agonistic activity of newly synthesized N-modified analogues of N/OFQ(1–13)NH_2 with aminophosphonate moiety was investigated for the first time.

Keywords Nociceptin analogues · Nociceptin/orphanin FQ · NOP receptor · SPPS · Aminophosphonates · Rat vas deferens

Introduction

Nociceptin/orphanin (N/OFQ) is an endogenous peptide, identified by Meunier et al. (1995) and Reinscheid et al. (1995), which binds selectively to its own receptors (NOP receptors), previously referred to as 'orphan' receptor. Nociceptin is a neuropeptide of 17 amino acids (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln), derived from a larger precursor, prepronociceptin (ppN/OFQ), which is similar in humans, rats and mice (Mollereau et al. 1996; Guerrini et al. 2000a, b, c). Despite its structural similarity to opioids, namely dinorphin A, N/OFQ does not interact with classical opioid receptors (Henderson and McKnight 1997; Darland et al. 1998; Guerrini et al. 2001a, b). This shows that the N/OFQ/NOP-system is a separate circuit with its own biological functions. Nociceptin is known mainly for its involvement in the transmission of pain signals and such physiological as nociception, locomotion, feeding behaviour, and respiratory, gastrointestinal and urogenital functions (Civelli, 2008; Pan et al. 2000; Dzambazova et al. 2008; Xie et al. 2008; Köster et al. 1999; Rizzi et al. 2001). Structure–activity relationship studies identified strategies to render N/OFQ ligands less susceptible to enzymatic degradation. These include truncation and C-terminal amidation, creating N/OFQ(1–13)NH_2 and modifications of the N-terminal region of N/OFQ as with [$Phe^1\psi(CH_2–NH)Gly^2$] N/OFQ(1–13)NH_2 (Guerrini et al. 1998, 2001a, b; Okada et al. 2000; Calo et al. 2000a, b; Kitayama et al. 2007; Arduin et al. 2007). Guerrini et al. (1998, 2000a, b, c, 2001a, b) have synthesized a series of analogues of the NOP-receptor antagonist [$Nphe^1$]-NC(1–13)NH_2 and have tested them for agonistic and antagonistic activities on the mouse vas deferens. The N-terminal tridecapeptide sequence of nociceptin molecule, N/OFQ(1–13)NH_2, was discovered to exert all the biological activities of N/OFQ, with arginine and lysine being crucial for the receptor binding (Naydenova et al. 2006). Identification of novel NOP-receptor ligands of either peptide or non-peptide nature has been the

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subject of intense research activity (Bignan et al. 2005; Zaveri 2003). The replacement of Ala with the Cα,α-dimethylated amino acid Aib at positions 7, 11 and 15 in N/OFQ sequence in the case of [Aib^7]N/OFQ-NH_2 produced a NOP-receptor ligand that was seven-folds more potent than the parent peptide, as determined in the [^{35}S]GTPcS functional assay (Zhang et al. 2002). Many analogues were also examined in search of variations of their functions, compared to the 'original' molecule (Rizzi et al. 2002).

Phosphonopeptides are phosphorus analogues of naturally occurring peptides containing a tetrahedral phosphorus atom. Their importance is obvious from the fact that they are being widely used as enzyme inhibitors and, as haptens in catalytic antibody research, because they can be considered as stable mimetics of tetrahedral transition states in ester and amide hydrolysis and formation (Hirschmann et al. 1994; Kafarski and Lejczak 2000a, b; Palacios et al. 2003; Cunningham et al. 2008). To date, several efficient synthetic routes have been developed for synthesis of phosphonopeptides and phosphinopeptides, containing C-terminal α-aminoalkylphosphinic acids (Kukhar et al. 1994; Soloshonok et al. 1992, 2010; Xu and Yu 1999; Meng and Xu 2010). As part of our research, the synthesis, the characterisation and the biological activity of new series of small peptides with aminophosphonate moiety as NOP-receptor ligands, have previously been described (Naydenova et al. 2010a, b).

Herein, we report the synthesis and biological activity of novel NOP-receptor ligands. The new N/OFQ(1–13)NH_2 derivatives with modification at 1st position by 1-[(methoxyphosphono)methylamino]cycloalkanecarboxylic acids were prepared using SPPS Fmoc-chemistry. In this study, we tested the new ligands in vitro on electrically stimulated smooth-muscle preparations from rat *vasa deferentia*. The biological activity of the new compounds and the relationship 'structure–activity' were discussed.

Materials and methods

Synthesis

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).

The solid-phase peptide synthesis by Fmoc (9-fluorenylmethoxy-carbonyl) chemistry was used to obtain new NOP-receptor ligands. 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 Fmoc-Arg(Pbf)-OH, Ser—as Fmoc-Ser(t-Bu)-OH, Thr—as Fmoc-Thr(t-Bu)-OH and Lys—as Fmoc-Lys(Boc)-OH. All coupling reactions were performed, using for amino acid/TBTU/HOBt/DIEA/resin a molar ratio of 3/2.9/3/6/1. A 20%-piperidine solution in *N*,*N*-dimethylformamide (DMF) was used to remove the Fmoc group at every step. The coupling and deprotection reactions were checked by the Kaiser test. We have prepared the corresponding aminophosphonates, according to recently published papers (Naydenova et al. 2008, 2010a, b). All the *1-[(dimethoxyphosphono)methylamino]cycloalkanecarboxylic acids* (3 equiv) were coupled to the growing peptide chain by using TBTU (3 equiv) in the presence of an equimolar concentration of 1-hydroxy benzotriazole (HOBt) dissolved in excess of *N*,*N*-diisopropylethylamine (DIEA) and DMF. The coupling reaction time was 15 h. 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. During the cleavage, one of the methoxy groups from cyclic aminophosphonic residue was removed. After filtration of the exhausted resin, the solvent was concentrated in vacuum and the residue triturated with cool ether. The purity of the peptides was checked by HPLC analysis, column: SymmetryShield^{TM} RP-18, 3.5 μ (50 × 4.6 mm), flow: 1 ml/min, H_2O (0.1% TFA)/CH_3CN (0.1% TFA), gradient 0 → 100% (15 min) and 100% (4 min). The crude peptides were purified using semi-preparative HPLC, column XBridge^{TM} Prep C18 10 μm (10 × 250 mm), flow: 5 ml/min, H_2O (0.1% TFA)/CH_3CN (0.1% TFA), gradient 20 → 70% (20 min). The ESI mass spectra were recorded with a platform II quadrupole mass spectrometer fitted with an electrospray source. Optical rotations were measured with a Perkin Elmer 341 polarimeter. Amino acid analysis was performed on a HITACHI L-8500 amino acid analyser on samples hydrolysed in 6 N HCl at 110°C for 24 h. Amino acid analysis of the peptide acid hydrolysates gave the expected amino acid ratios. The analytical data of the synthetic peptides prepared were as follows: compound **NOC-1** t_R 4.10 min, >98% pure, 1,452.6 calculated (MH^+), 1,452.8 observed (MH^+); compound **NOC-2** t_R 4.60 min, >99% pure, 1,599.8 calculated (MH^+), 1,599.5 observed (MH^+); compound **NOC-3** t_R 4.40 min, >99% pure, 1,480.8 calculated (MH^+), 1,480.3 observed (MH^+); compound **NOC-4** t_R 5.10 min, >98% pure, 1,627.8 calculated (MH^+), 1,627.5 observed (MH^+); compound **NOC-5** t_R 4.80 min, >98% pure, 1,494.8 calculated (MH^+), 1,494.9 observed (MH^+); compound **NOC-6** t_R 5.20 min, >98% pure, 1,641.9 calculated (MH^+), 1,641.7 observed (MH^+).

Biological tests

The biological activity of the newly synthesized analogues of N/OFQ (1–13)NH_2 was tested in vitro on electrically

stimulated preparations of rat vas deferens. The smooth muscles were isolated from Wistar albino rats, weighing 180–200 g. The prostatic parts of the *vasa deferentia*, 12–15 mm long, were carefully cleaned from the surrounding connective tissues and blood vessels, fixed in 4-ml organ bath and connected to electronic transducers. Before the experiment, the preparations were preloaded with 1 g and left for a 60-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 stimulation (LFES) with parameters 0.05 Hz frequency, 1 ms pulse duration, sub-maximal voltage. The smooth-muscle tone and the electrically induced contractions were isometrically registered. The tested compounds were cumulatively applied to the organ bath in concentrations of 1×10^{-11} M–1×10^{-5} M. In some experiments, 1×10^{-6} M naloxone (to block μ-, δ- and κ- opioid receptors) or 3×10^{-5} M naloxone benzoylhydrazone (to block opioid and nociceptin receptors) was administered 10 or 15 min, respectively, before the examined compounds.

In some experiments, cumulative concentration–response curves of N/OFQ(1–13)NH_2 were built in the absence or in the presence of **NOC-3** (in three different concentrations—1×10^{-7}, 1×10^{-6} and 1×10^{-5} M), applied 30 min before **N/OFQ(1–13)NH_2**. In order to protect the peptides from degradation, a cocktail of peptidase inhibitors (amastatin, bestatin, phosphoramidon and captopril (Nicholson et al. 1998)) in a final concentration of 3×10^{-5} M, was added to the incubation medium.

Animals

In the experiments, male Wistar rats (180–200 g), housed at 22–25°C, were used. The animals were allowed an adaptation period with free access to food and water, and a natural day/night light cycle. All the animals were killed under light ether anaesthesia.

The experiments were performed according to the requirements of the European Convention for the Protection of Experimental Animals (Protection of animals used for experimental purposes, Council Directive 86/609/EEC of November 1986) and the rules of the Ethics Committee of the Institute of Neurobiology, BAS.

Results and discussion

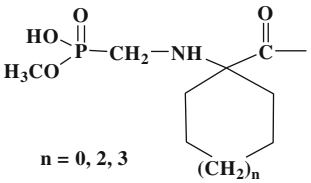
Chemistry

We have recently reported structure–activity relationship of hexapeptides analogues with aminophosphonates moiety as NOP-receptor ligands (Naydenova et al. 2010a, b). Now, we prepared and evaluated for agonistic activity in vitro new N-modified analogues of the N/OFQ(1–13)NH_2 with aminophosphonate moiety containing five-, seven- and eight-membered cycloalkane rings (Table 1). The peptides (**NOC 1–NOC 6)** were obtained with good yield by solid phase peptide synthesis—Fmoc (9-fluorenylmethoxy-carbonyl) chemistry, according to the previously described procedure (Naydenova et al. 2010a, b). The optical rotations and amino acid analysis data of the newly synthesized compounds are shown in Table 1. The 1-[(dimethoxyphosphono) methylamino]cycloalkanecarboxylic acids were previously prepared using Kabachnik-Fields reaction (Naydenova et al. 2008, 2010a, b).

In order to estimate the 'structure–activity' relation, we prepared the compounds **NOC-1, NOC-3,** and **NOC-5** by replacement of the N-terminal Phe in N/OFQ(1–13)NH_2 with 1-[(methoxyphosphono)methylamino]cycloalkanecarboxylic acids. Aiming to additionally prove the importance of Phe, we introduced the 1-[(methoxyphosphono)methylamino] cycloalkanecarboxylic acids to the N-side of N/OFQ(1-13) NH_2 **(NOC-2, NOC-4, NOC-6)**. The new analogues are with the following sequences:

X-GGFTGARKSARK-NH_2
(NOC-1, NOC-3, NOC-5)
X-FGGFTGARKSARK-NH_2
(NOC-2, NOC-4, NOC-6)

where ***X*** is:

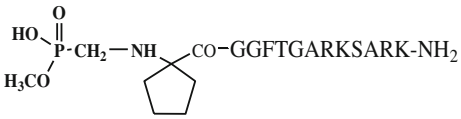
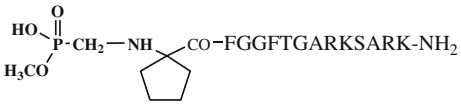
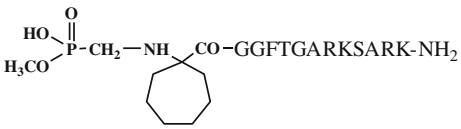
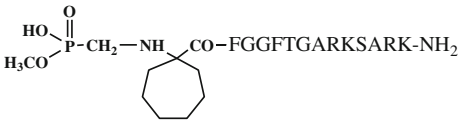
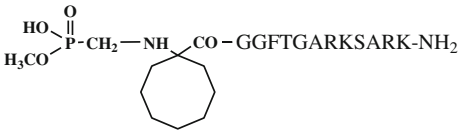
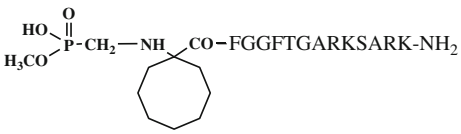


The crude peptides were purified on a reversed-phase high-performance liquid chromatography (HPLC) and the molecular weights were determined, using electrospray ionisation mass-spectrometry. The analytical data are shown in the "Materials and methods".

Biological activity

Intensive structure–activity studies (Dooley et al. 1997; Reischeid et al. 1996; Butour et al. 1997; Guerrini et al. 2000a, b, c) have shown that the sequence Phe^1-Gly–Gly-Phe^4 is critical for the message domain of N/OFQ, involved in receptor binding and activation. The newly synthesized peptides, tested in our laboratory, could be divided in two groups. In the first one, aminophosphonate substituents with varying ring (C5, C7, C8) are linked to Phe^1 and the indicated sequence remains intact. The peptides, including the natural sequence (**NOC-2**, **NOC-4**, **NOC-6**), exert very similar agonist activity (pEC50 = 6.17–6.51) and strong

Table 1 Physicochemical data for synthetic peptides

No.	Peptides	$[\alpha]_D^a$ (°)	Amino acid analysis[b]						
			Gly	Phe	Thr	Ala	Arg	Ser	Lys
NOC-1		−17.5	3.07	1.04	1.03	2.04	2.00	1.04	2.04
NOC-2		−22.6	3.06	2.00	1.07	2.08	2.04	1.00	2.02
NOC-3		−16.9	3.00	1.09	1.00	2.02	2.08	1.06	2.00
NOC-4		−21.7	3.01	2.10	1.06	2.01	2.10	1.06	2.01
NOC-5		−18.1	3.05	1.10	1.00	2.00	2.00	1.04	2.00
NOC-6		−23.2	3.08	2.07	1.07	2.05	2.10	1.10	2.02

[a] Optical rotation in 1% AcOH ($c = 1$) at 20°C

[b] After hydrolysis with 6 N HCl at 110°C for 24 h. The corresponding *1-[(dimethoxyphosphono)methylamino]cycloalkanecarboxylic acids* do not give ninhydrin detection and cannot be proved by this method

inhibition of smooth-muscle contractions ($E_{max} = -70$ to -90%, comparable to that of N/OFQ(1–13)NH_2). In this group (and for the whole study), **NOC-6** is the most active compound (Fig. 1). Unexpectedly, the larger cycle (C8) in **NOC-6** increased the activity and efficacy of the compound, compared to peptides **NOC-2** and **NOC-4**. Naloxone had no effect, whilst the pre-incubation of the preparations with naloxone benzoylhydrazone completely prevented the inhibition of smooth-muscle contractions, thus showing that these three modified peptides selectively bound to NOP receptors (Figs. 1, 2, 3).

When Phe at position 1 is substituted by the same aminophosphonate residues (**NOC-1**, **NOC-3**, **NOC-5**), the obtained derivatives showed much lower activity and affinity in the experiments on electrically induced smooth-muscle contractions (Table 2). The effects are prevented to a great extent by pre-incubation of the smooth-muscle preparations with naloxone (Figs. 4, 5, 6); this indicates that these new derivatives have gained affinity for classical opioid receptors. Based on structure–activity relationship study of N/OFQ, Reinscheid et al. (1996) have suggested

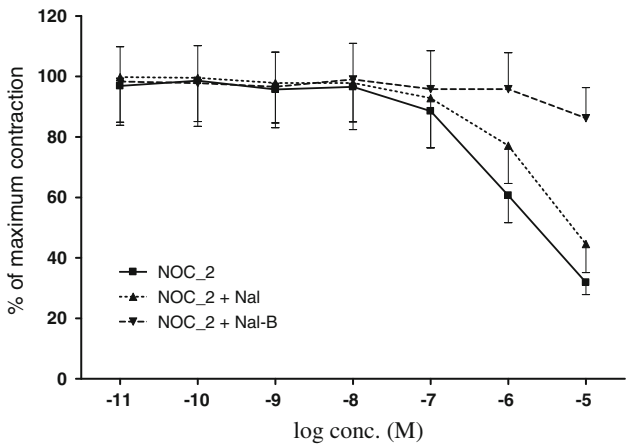


Fig. 1 Effects of N/OFQ(1-13)NH_2 and **NOC-6** on LFES-induced contractions

that more bulky side chain in this position prevents the formation of a favourable secondary structure. In the derivatives of the second group, a very big side chain is

linked to Gly^2, which could be the possible reason for the low activity. The replacement of Phe at position 1 by 1-[(methoxyphosphono)methylamino]cycloalkanecarboxylic acids goes along with a loss of receptor affinity and biological activity of the obtained derivatives (Table 2, Figs. 4, 5, 6).

The observed reduced efficacy (E_{max}) of the compounds in this group gave us reason to test the antagonistic

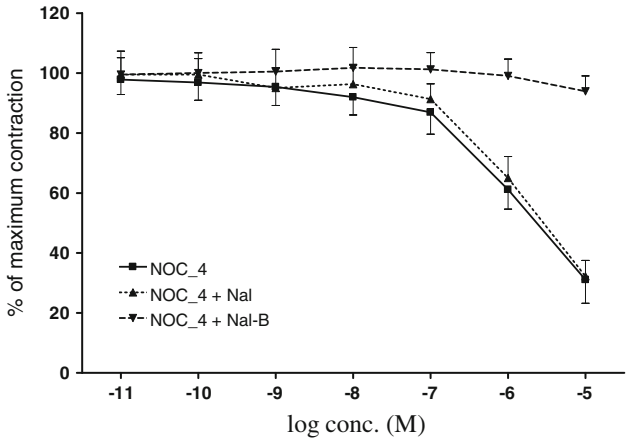


Fig. 2 Effects of **NOC-2** on LFES-induced contractions

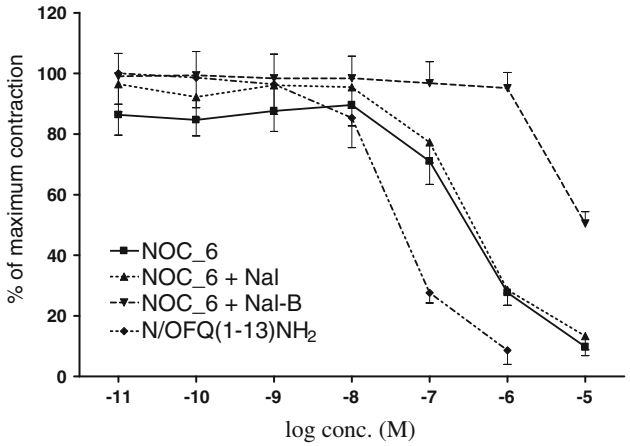


Fig. 3 Effects of **NOC-4** on LFES-induced contractions

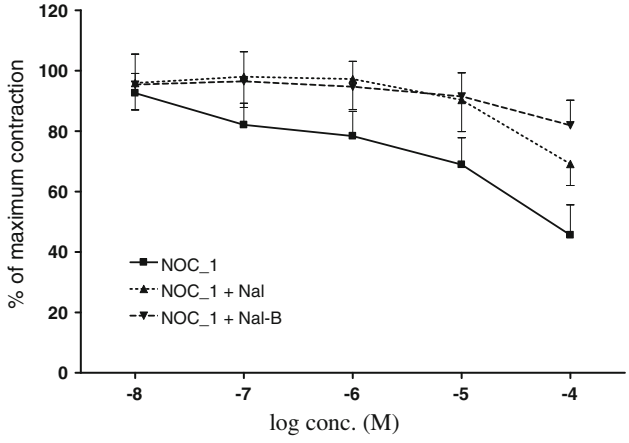


Fig. 4 Effects of **NOC-1** on LFES-induced contractions

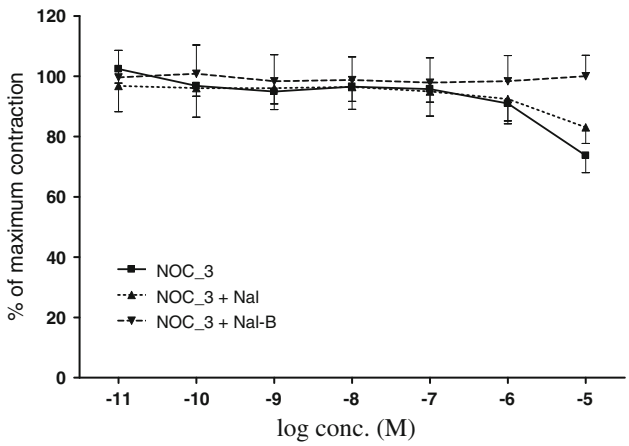


Fig. 5 Effects of **NOC-3** on LFES-induced contractions

Table 2 Biological activity of the newly synthesized peptides on LFES-evoked contractions of rat vas deference

Peptide tested	Peptide alone		Peptide + naloxone	
	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)
N/OFQ(1-13)NH_2	7.42 ± 0.11	−85 ± 2	7.18 ± 0.13	−86 ± 3
NOC-1	5.37 ± 0.31	−54 ± 10	4.85 ± 0.72	−31 ± 7
NOC-2	6.17 ± 0.12	−68 ± 4	5.92 ± 0.08	−55 ± 9
NOC-3	6.28 ± 0.33	−26 ± 6	5.83 ± 0.36	−17 ± 5
NOC-4	6.16 ± 0.13	−69 ± 8	6.06 ± 0.06	−68 ± 5
NOC-5	5.92 ± 0.17	−48 ± 10	5.74 ± 0.72	−19 ± 8
NOC-6	6.51 ± 0.15	−90 ± 3	6.55 ± 0.10	−87 ± 5

The maximal inhibitory effect of N/OFQ(1–13)NH_2 is reached at a concentration of 10^{-6} M. Compared to N/OFQ(1–13)NH_2, the newly synthesized analogues have shown much lower activity. With them, the initial effect, as well as the maximal one, developed at higher concentrations. Therefore, in Table 2, the equi-effective maximal concentrations have been used

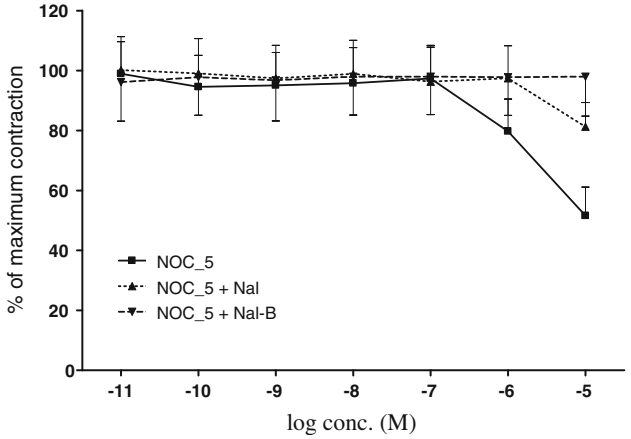


Fig. 6 Effects of **NOC-5** on LFES-induced contractions

properties of **NOC-3** on the response to N/OFQ(1-13)NH_2 (data not shown). In the presence of **NOC-3** in any of the concentration tested (1×10^{-7}, 1×10^{-6}, 1×10^{-5} M), there was no concentration-dependent rightward shift of the nociceptin response curve, indicating that the peptide has no inhibitory properties but rather is a very week agonist.

We describe the synthesis and biological investigation of novel N-modified analogues of the N/OFQ(1–13)NH_2 with aminophosphonate moiety. The newly synthesized compounds were obtained by solid-phase peptide synthesis—Fmoc-strategy. All of the peptides were tested for agonistic activity in vitro. Our results have shown that the selectivity of the peptides containing 1-[(methoxyphosphono)methylamino]cycloalkanecarboxylic acids at the N-side of Phe is not changed—they remain selective agonists of NOP receptors. The derivative with the largest ring (**NOC-6**) demonstrated efficacy similar to that of N/OFQ(1–13)NH_2, but in a 10-fold higher concentration. Attachment of aminophosphonates to Gly^2 goes along with a loss of biological activity of the obtained derivatives and an expressed affinity to the classic opioid receptors.

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