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ORIGINAL ARTICLE

Synthesis and biological activity of novel small peptides with aminophosphonates moiety as NOP receptor ligands

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Abstract The aim of the present study was the synthesis and the biological screening of new analogs of Ac-RY-YRWK-NH_2, modified at the N-terminal with 1-[(methoxyphosphono)methylamino]cycloalkanecarboxylic acids. The four newly synthesized ligands for the nociceptin/orphanin FQ (N/OFQ) receptor (NOP) have been prepared by solid-phase peptide synthesis—Fmoc-strategy. These compounds were tested for agonistic activity in vitro on electrically stimulated smooth-muscle preparations isolated from vas deferens of Wistar rats. Our data showed that substitution of Arg at position 1 with aminophosphonates moiety decreased significantly the affinity of ligands to the NOP receptor. Furthermore, the enlargement of the cycle (with 5–8 carbon atoms) additionally diminished both the activity and the selectivity for NOP-receptor.

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

Introduction

Nociceptin/Orphanin FQ (N/OFQ) is a heptadecapeptide (Meunier et al. 1995; Reinscheid et al. 1995), which modulates a number of biological functions in the central and the peripheral nervous system (Calo et al. 2000; Mogil and Pasternak 2001; Dzhambazova et al. 2008) by selectively activating an opioid-like receptor named ORL_1 or N/OFQ peptide (NOP) receptor (Cox et al. 2000), a novel member of the opioid receptor family.

By selective activation of G-protein coupled receptor (NOP), N/OFQ produces inhibition of adenylate cyclase (Meunier et al. 1995; Reinscheid et al. 1995), activation of inward K^+ current (Matthes et al. 1996), and inhibition of Ca^{2+} channels (Connor et al. 1996). Moreover, recent data indicate that NOP antagonists are worthy of developing as innovative antiparkinson drugs (Marti et al. 2005). Hence, the NOP receptor system is highly attractive molecular target for drug development purposes (Zaveri 2003). The understanding of the role of the N/OFQ/NOP system depends upon the development of selective and highly potent peptide and non-peptide agonist and antagonist ligands. Among them are the N/OFQ related peptides (Guerrini et al. 2000; Naydenova et al. 2006; Arduin et al. 2007) and small peptides, identified by screening of peptide combinatorial libraries (Dooley et al. 1997).

Arduin et al. reported the synthesis and biological evaluation of a series of N/OFQ-NH_2 analogs substituted in position 7 and 11 with Cα,α,-disubstituted cyclic, linear, and branched amino acids compatible with alpha helix structures (Arduin et al. 2007). The most interesting results in terms of biological activity have been obtained using Aib in position 7. This substitution was then combined with other chemical modifications and series of novel highly potent peptide ligands for the NOP receptor were generated. This receptor is also activated by non-peptide agonists (Jenck et al. 2000). Recently, a potent and selective non-peptide antagonist has been reported (Noda et al. 1998; Ozaki et al. 2000), but it has been difficult to identify any general structural elements, common to all of these organic compounds.

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Dooley et al. screened a synthetic peptide combinatorial library, and isolated and characterized several hexapeptides, including Ac-RYYRWR-NH_2, Ac-RYYRWK-NH_2 and Ac-RYYRIK-NH_2, which have high affinity for the NOP receptor. These hexapeptides are not only significantly smaller than N/OFQ-[1-13]-NH_2, the shortest N/OFQ fragment with the same affinity and biological potency as the parent peptide, but they also exhibit distinctive pharmacological profiles (Calo et al. 2000). The hexapeptide Ac-RYYRWK-NH_2 has been described as a potent partial agonist of the NOP receptor, without affinity to μ-, κ- or δ- opioid receptors (Dooley et al. 1997). In vivo, Ac-RYYRWK-NH_2 has been shown to increase food intake in rats similarly to N/OFQ, and to mimic N/OFQ in mouse colon preparation (Mason et al. 1998; Rizzi et al. 1999). In particular, the hexapeptide Ac-RYYRIK-NH_2 has been shown to competitively inhibit nociceptin (NC)-induced guanosine-5′-*O*-(3-thio)trisphosphate-γ-S (GTPγS) binding to the NOP receptor (Berger et al. 1999, 2000). These hexapeptides contain three positively charged basic residues similar to N/OFQ, which has four positively charged amino acids at the C-terminus. In an Ala-scanning study, two Arg residues, at positions 1 and 4, were found to be important for binding to the NOP receptor (Kawano et al. 2002).

Phosphonopeptides and phosphinopeptides are phosphorus analogs of naturally occurring peptides. Their importance is obvious from the fact that they have been widely used as enzyme inhibitors and as happens for catalytic antibody research because they can be considered as stable mimetics of tetrahedral transition states in ester and amide hydrolysis and formation (Kafarski and Lejczak 2000a, b; Cunningham et al. 2008). Many methods are now available for preparation of phosphinopeptides, containing C-terminal α-aminoalkylphosphinic acids (Kukhar et al. 1994; Soloshonok et al. 1992; Xu and Yu 1999; Meng and Xu 2010). Presently, there are no data about synthesis and biological activity of peptides containing N-terminal 1-[(methoxyphosphono)methylamino]cycloalkanecarboxylic acids.

Based on Ac-RYYRWK-NH_2 as a chemical template, we synthesized novel NOP-receptor short-chain analogs modified at N-terminal with α-aminocycloalkane carboxylic acid, bearing (methoxyphosphonyl)methyl group. Besides, we investigated the structure–activity relationship of the new analogs in vitro on electrically stimulated smooth-muscle preparations from rat *vas deferens*.

Materials and methods

Synthesis

The protected amino acids and Fmoc-Rink Amide MBHA Resin 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-fluorenylmethoxycarbonyl) chemistry was used to obtain nociceptin/orphanin FQ ligands. Rink-amide resin was used as a solid-phase carrier, and TBTU was used as a coupling reagent. The 3-functional amino acids were embedded as follows: Arg—as Fmoc-Arg(Pbf)-OH, Lys—as Fmoc-Lys(Boc)-OH, Trp—as Fmoc-Trp(Boc)-OH, Tyr—as Fmoc-Tyr(tBu)-OH. The coupling reactions were performed using for amino acid/TBTU/HOBt/DIEA/resin a molar ratio of 3/2.9/3/6/1, in a 1:1 mixture of DMF/DCM. Piperidine in dimethylformamide (20% solution) was used to remove the Fmoc group at every step. The coupling and deprotection reactions were checked by the Kaiser test. All the 1-[(dimethoxyphosphono)methylamino] cycloalkanecarboxylic acids (3 equiv) were coupled to the growing peptide chain by using 2-(1H-Benzotriazole-1-yl)1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 3 equiv) in the presence of an equimolar concentration of 1-hydroxy benzotriazole (HOBt), dissolved in N,N-dimethylformamide (DMF) at excess of N,N-diisopropylethylamine (DIEA), the coupling reaction time being 15 h. The cleavage of the synthesized peptides from the resin was done, using a mixture of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), and 2.5% water. After filtration of the exhausted resin, the solvent was concentrated in vacuum and the residue triturated with cool ether. The peptides were analyzed on a reversed-phase high-performance liquid chromatography (HPLC) C18 column: flow 1 ml/min, H_2O/CH_3CN/0.1% TFA gradient 0 → 100% 20 min. The peptide purity was checked by electrospray ionization massspectrometry. The analytical data for the synthesized peptides prepared were as follows: compound **3** t_R 5.40 min, >98% pure, 1032.5 calculated (MH^+), 1033.0 observed (MH^+); compound **4** t_R 5.60 min, >99% pure, 1060.5 calculated (MH^+), 1060.8 observed (MH^+); compound **5** t_R 5.80 min, >99% pure, 1074.5 calculated (MH^+), 1075.0 observed (MH^+); compound **6** t_R 5.06 min, >99% pure, 1203.6 calculated (MH^+), 1203.1 observed (MH^+).

Biological tests

The biological activity of the newly synthesized peptides has been tested on electrically stimulated smooth-muscle preparations. The dissected *vasa deferentia* were cleaned from the surrounding tissues and blood vessels. Prostatic segments (12–15 mm long) were fixed in 4 ml organ baths and connected to electronic transducers. The preparations, pre-loaded with 1 g, were left for a 60 min adaptation at 32.5°C in carbogen-aerated Krebs solution. Smooth-muscle contractions were evoked by low-frequency electrical stimulation with parameters: 0.05 Hz frequency, 1 ms pulse duration, sub-maximal voltage.

The smooth-muscle tone of the ES-induced contractions was registered by on-line system. The tested compounds were applied cumulatively in concentrations 10^{-10}–10^{-4} M. In part of the experiments naloxone (NaI, 10^{-6}M, for to block all types opioid receptors, IUPHAR-DB) or naloxone benzoylhydrazone (3×10^{-5}M, as a blocker of NOP receptors, Chiou 2001) was administered to the organ bath 10 or 15 min prior to the peptides under investigation.

All compounds were dissolved in saline prior the experiment. Ac-RYYRWK-NH_2 was dissolved in 0.01 M acetic acid.

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 animals were killed under light ether anesthesia.

The experiments were performed according to the "Principles of laboratory animals care" (NIH publication No 85–23, revised 1985) and the rules of the ethic Committee of the Institute of Neurobiology, BAS.

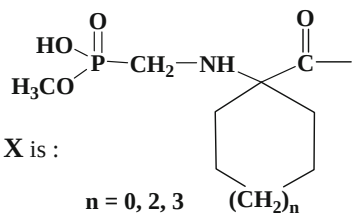
Results and discussion

Chemistry

The peptides shown in Table 1 were prepared with good yield by solid phase synthesis using TBTU, an efficient peptide coupling reagent. The 1-[(dimethoxyphosphono) methylamino]cycloalkanecarboxylic acids were previously prepared by our group following Kabachnik- Fields reaction (Naydenova et al. 2008, 2010). In view of the low solubility of α-aminophosphonates, the coupling reaction was performed in *N*,*N*-dimethylformamide (DMF) at high excess of *N*,*N*-diisopropylethylamine (DIEA) and the coupling reaction time was 15 h. During the TFA cleavage one of the methoxy groups from aminophosphonic residue was removed. In compounds **3**–**5**, Arg at position 1 was substituted by 1-[(methoxyphosphono) methylamino] cycloalkanecarboxylic acids.

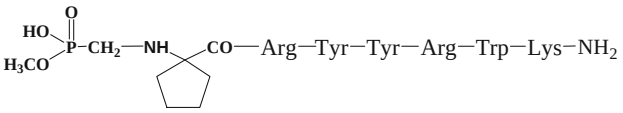
The new hexapeptides have the following sequences:

X–Tyr–Tyr–Arg–Trp–Lys–NH_2



where **X** is :

In order to elucidate the influence of Arg and acetyl group we decided to introduce the 1-[(methoxyphosphono)methylamino]cyclopentanecarboxylic acid to the N-side of Arg, compound **6**. This newly synthesized heptapeptide has the following sequence:



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

Biological activity

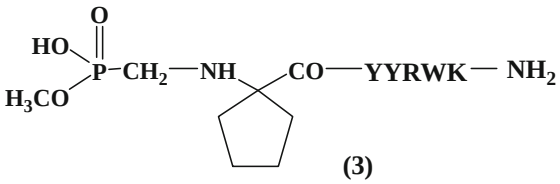
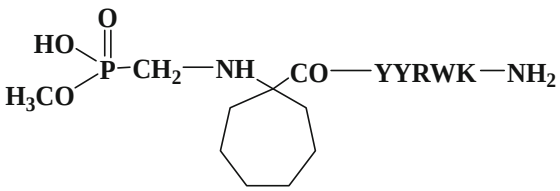
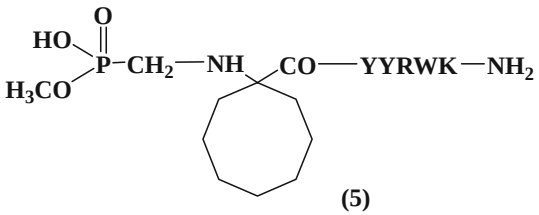
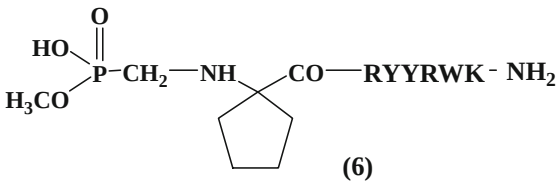
All newly synthesized compounds induce a strong tachyphylaxis (similarly to Ac-RYYRWK-NH_2; Dooley et al. 1997). That is why each cumulative dose–response curve for the peptides (without or with blockade of opioid and nociceptin receptors) was created on a separate vas-deferens preparation.

The effects of newly synthesized short-chain peptides (**3**–**6**) were compared with Ac-RYYRWK-NH_2 (**1**), which is known to be a very potent and selective partial agonist of NOP receptors (Dooley et al. 1997). Therefore, it has been synthesized in our laboratory as a referent compound. Tested on rat vas deferens, data ($pEC_{50} = 8.9$; $E_{max} = -40\%$) similar to those reported in the literature ($pEC_{50} = 9.1$; $E_{max} = -66\%$; Ho et al. 2000) were obtained.

The removal of the acetyl group in the parent peptide (**2**) dramatically reduced both the potency ($pEC_{50} = 4.84$) and efficacy ($E_{max} = -25\%$, Fig. 1). Furthermore, the replacement of the acetyl group by aminophosphonates moiety (compound **6**, Fig. 2) also reduced the potency of the peptide ($pEC_{50} = 5.08$). Nevertheless, its action remains specific for NOP receptors: applied after opioid receptor blockade by naloxone, the peptide does not change its effect. The blockade of NOP receptors with naloxone benzoylhydrazone prevented completely the inhibitory effect of compound **6**.

In the three hexapeptide derivatives (**3**, **4**, and **5**), Arg^1 was substituted by 1-[(methoxyphosphono)methylamino] cycloalkanecarboxylic acids with 5-, 7-, and 8-membered rings. The enlargement of the cycle (compounds **4** and **5**) significantly diminished pEC_{50} and E_{max}. Practically, these two compounds affect the smooth-muscle contractions only in the highest concentration used (10^{-4}M, Fig. 3), most probably not by interactions with NOP receptors. In relatively low concentrations (10^{-8}–10^{-7}M), compound **3**

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

Peptide tested	Peptide alone		Peptide + Nal		Peptide + Nal-B	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Ac—RYYRWK—NH_2 **(1)**	8.95	−40.0	9.37	−44.7	6.8	−13.9
H—RYYRWK—NH_2 **(2)**	4.84	−25.0	4.94	−7.7	4.92	−3.5
 **(3)**	5.82	−16.2	4.83	−7.67	5.00	−4.75
 **(4)**	4.81	−7.4	4.86	−9.44	4.52	1.32
 **(5)**	4.78	−3.25	4.85	−5.63	4.71	−0.4
 **(6)**	5.08	−25.5	5.19	−23.5	4.74	−2.6

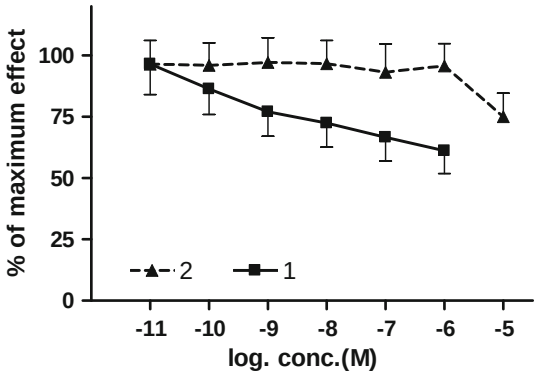


Fig. 1 Effects of Ac-RYYRWK-NH_2 (**1**) and H-RYYRWK-NH_2 (**2**) on LFES-induced smooth-muscle contractions

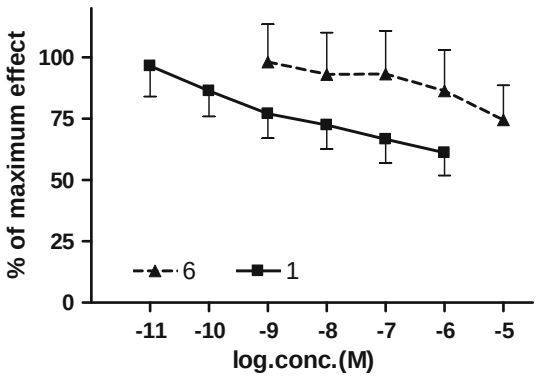


Fig. 2 Effects of hexapeptide analogs (**1** and **6**) on LFES-induced smooth-muscle contractions

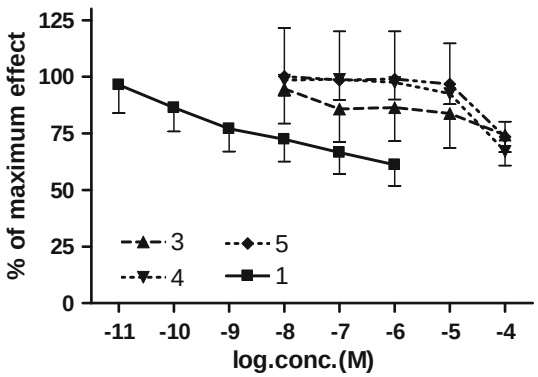


Fig. 3 Effects of Ac-RYYRWK-NH_2 (**1**) and the hexapeptide analogs (**3**–**5**) on LFES-induced smooth-muscle contractions

(in the molecule of which a 1-[(methoxyphosphono)methylamino]cyclopentanecarboxylic residue is included) moderately decreased the contractions of vas deference preparations, as compared with compound **1**.

The N-terminal modification with aminophosphonates moiety decreased the affinity of the ligands to NOP receptor. The same effect has been shown for N-terminal modifications with other groups (pyvaloyl, formyl, benzoyl, mesyl) in an investigation of Gündüz et al. (Gündüz et al. 2006). Our results lead also to the conclusion that when Arg^1 is included in the hexapeptide molecule, the affinity and selectivity for NOP receptor is preserved (compound **6**). It might be suggested that the smooth-muscle inhibition by compounds **3**–**5** (where Arg^1 is replaced) is due to the interaction of these peptide analogs with the classical opioid receptors and not with the NOP-receptor. Dooley et al. established that the following sequence Arg-Tyr-Tyr is the most effective at the first three positions of NOP-receptor ligands (Dooley et al. 1997). Later on, structure–activity studies on hexapeptides with modified amino acids confirm that Arg^1 is preferred and even "crucial" (Gündüz et al. 2006); moreover, it has been shown that both Arg^1 and Arg^4 in the hexapeptide chain are required for biological activity (Judd et al. 2004).

It is known that the activation of all opioid receptors requires a Tyr residue in position 1. There are many data from structure–activity studies, showing that replacement of Phe^1 in nociceptin molecule with Tyr produces a peptide, which activates NOP receptor (Butour et al. 1997; Varani et al. 1998), as well as opioid μ (Salvadori et al. 1997) and κ (Reinscheid et al. 1998) receptors. However, the shorter nociceptin analogs [Tyr^1]NC(1–9)NH_2 and [Tyr^1]NC(1–5)NH_2 activate the classical opioid receptors only (Varani et al. 1999).

Concerning compound **3**, it should be stated that the inhibitory effects of the compound after naloxone and naloxone benzoylhydrazone are practically equal. This suggests that compound **3** most probably is agonist of opioid receptors only, since its activity is blocked by naloxone, being not further affected by naloxone benzoylhydrazone.

Our results suggest that the incorporation of 1-[(methoxyphosphono)methylamino] cycloalkanecarboxylic acid in position 1 of Ac-RYYRWK-NH_2 decreases the N/OFQ-like activity of the newly synthesized peptide analogs.

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