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Synthesis and electrophysiological characterization of cyclic morphiceptin analogues

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Abstract

A challenge in opioid peptide chemistry and pharmacology is the possibility to develop novel peptides with peripheral selectivity. An enzymatically stable opioid peptide could involve an antidiarrheal effect. For this reason, we constrained the highly selective and potent tetrapeptide morphiceptin with a 6-atom bridge, resulting in a cyclic amide and an ester analogue, **2** and **3**, respectively.

Taking advantage of the functional coupling of the opioid receptor with the heteromultimeric G-protein-coupled inwardly rectifying K^+ (GIRK1/GIRK2) channel, either the wild-type μ-, κ-, δ- or a mutated μ-opioid receptor (hMORS329A) was functionally co-expressed with GIRK1/GIRK2 channels and a regulator of G-protein signaling (RGS4) in *Xenopus laevis* oocytes. The two-microelectrode voltage clamp technique was used to measure the opioid receptor activated GIRK1/GIRK2 channel responses. Both cyclic analogues were equally potent via the wild-type μ-opioid receptor hMORwt (EC_{50} value 976.5 ± 41.7 for **2** and 1017.7 ± 60.7 for **3**), while the EC_{50} value for Tyr-Pro-Phe-D-Pro-NH_2 measured 59.3 ± 4.8 nM. These three agonists displayed a four to five times decreased potency via hMORS329A as compared to the wild type. Interestingly, no effect on κ- and δ-opioid receptors was observed. The intramolecular bridge created by cyclization of morphiceptin prevents dipeptidyl peptidase IV from interacting with these analogues.

We conclude that constraining morphiceptin with a 6-atom bridge resulted in enzymatically stable peptidomimetics that are exclusively active on μ-opioid receptors. These analogues provide an interesting template in the promising approach for the design of potential antidiarrheal agents.

Keywords: Cloned opioid receptors; *Xenopus* oocytes; Morphiceptin analogues; Voltage clamp; RGS; GIRK

1. Introduction

Opioid receptors are activated by two main kinds of molecules. The first group comprises the opiates, the non peptide molecules like morphinans, benzomorphinans, phenylpiperidines, and oripavines [1], the second group consists of the opioid peptides. Two types of endogenous opioid peptides exist, one containing Tyr-Gly-Gly-Phe as the message domain (enkephalins, endorphins, dynorphins) and the other containing the Tyr-Pro-Phe/Trp sequence. This latter group comprises the highly selective endogenous μ-opioid receptor ligands endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) that produce potent and prolonged analgesia in test animals [2].

Morphiceptin (Tyr-Pro-Phe-Pro-NH_2), a synthetic tetrapeptide amide with a similar sequence to the endomorphins, is a potent and selective agonist at the μ-opiate receptor. This peptide was originally synthesized as an analogue possessing the N-terminal tetrapeptide fragment of β-casomorphin. Morphiceptin is about 50–100 times more active than β-casomorphin in receptor binding assays and in the guinea pig ileum test. Its potency in the mouse vas deferens assay and its affinity for δ-receptors are much lower [3].

Besides the central role of opiates in analgesia, morphiceptin analogues have also been proposed as peripheral

Abbreviations: Boc, *t*-butoxycarbonyl; BOP, benzotriazole-1-yloxy-tris(dimethylamino)phosphoniumhexafluorophosphate; Fmoc, fluoren-9-ylmethylsuccinimidyl carbonate; 32 GIRK1/GIRK2, G-protein-coupled inwardly rectifying potassium channel; hDOR, human wild-type δ-opioid receptor; hKOR, human wild-type κ-opioid receptor; hMOR, human wild-type μ-opioid receptor (hMOR); RGS4, regulator of G-protein signaling; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate

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agents. Firstly, morphiceptin analogues showed antidiarrheal activity. Intracerebroventricular or subcutaneous administration of Tyr-Pro-NMePhe-D-Pro-NH_2, a potent morphiceptin derivative, inhibited diarrhea and decreased the gastrointestinal transit [4]. This observation provides an interesting approach to the development of peripheral opioid receptor agonists as antidiarrheal agents. These drugs would lack the central effects of analgesia and sedation, and would not cause constipation. On the other hand, morphiceptin and other β-casomorphins inhibited cell proliferation of human prostate cell lines, by a mechanism partly involving opioid receptors [5,6]. Very recently, Janecka et al. [7] presented a series of new morphiceptin analogues, modified at the pharmacophoric position 3 and their binding to μ-opioid receptors in experimental mammary adenocarcinoma. These therapeutical possibilities encourage further studies on this group of opioids, focusing on peripheral selectivity.

Since the discovery that morphiceptin is one of the most selective agonists for the μ-receptor, several structure–activity studies of this tetrapeptide have been carried out [8,9]. They modified morphiceptin by substitutions at the second, third, and fourth amino acid residues. Substitution of the fourth amino acid, L-Proline with D-Proline was the most effective. This analogue (Tyr-Pro-Phe-D-Pro-NH_2, **1**), relative to morphiceptin, is about 15 times more active on μ-receptor binding (Fig. 1).

In this study, we present two new cyclic analogues of morphiceptin, **2** and **3**, respectively (Fig. 1). These compounds were designed using NMR data and molecular modeling. Yamazaki et al. defined some main topochemical requirements for the μ-opioid receptor [10]. A probable bioactive conformation for a potent μ-agonist Tyr-Pro-NMePhe-D-Pro-NH_2 was proposed. Looking for bridging units to constrain D^4-morphiceptin **1** (Tyr-Pro-Phe-D-Pro-NH_2), we used their data to determine a cyclic bioactive conformer. Systemic conformational energy search was carried out with the Newton–Raphson method until the maximum derivative was less than 0.001 kcal mol^{-1} using Sybyl 6.5 (MIPS3-IRIX6.2). Conformational energies were estimated as the sum of nonbonded Van der Waals interactions, Coulombic interaction, intrinsic torsional potentials, and energies of deformation of bond lengths and bond angles. After energy minimization, 10 conformers with minimum energy were selected. The geometry optimization for the selected conformers was performed with the semi-empirical AM1 and PM3 method [11] employing MOPAC 6 software [12]. The minimum energy conformation showed great similarity with the conformation described by Yamazaki, adopting a *cis* Tyr-Pro amide bond, a trans conformation for the side chains of Tyr and Phe, and characteristic distances between the aromatic rings.

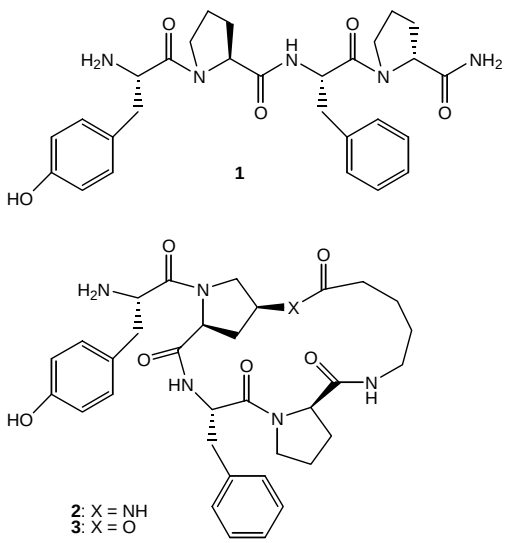

Fig. 1. Chemical structures of peptides **1**, **2**, and **3**.

These data allowed us to design a 6-atom bridge between the terminal amide group and the Pro^2-4 position, in order to provide a rigid cyclic peptide. A global minimum energy structure was deduced for Tyr-Pro-Phe-D-Pro-NH_2 in which Pro^2 was replaced by (*S*)-4-hydroxyproline, where the 4-hydroxy group is linked through a 5-C chain to the terminal amide. For the binding of the bridging unit with this hydroxygroup, we used an amide and an ester bond, as shown in structures **2** and **3** (Fig. 1).

To investigate the effect of this new cyclization on the potency of the tetrapeptide we performed an electrophysiological characterization of these products. Taking advantage of the functional coupling of the opioid receptor with the heteromultimeric G-protein-coupled inwardly rectifying potassium channel (GIRK1/GIRK2), either the human wild-type μ-opioid receptor (hMOR), the human wild-type κ-opioid receptor (hKOR), the human wild-type δ-opioid receptor (hDOR), or the mutated receptor (hMORS329A) was functionally co-expressed with GIRK1/GIRK2 channels together with a regulator of G-protein signaling (RGS4) in *Xenopus laevis* oocytes. The two-microelectrode voltage clamp technique was used to measure the opioid receptor activated GIRK1/GIRK2 channel responses, and from that the EC_{50} values were obtained. Co-expression of RGS4, the brain-expressed isoform of RGS proteins [13,14], reconstitutes the native gating kinetics by accelerating GIRK1/GIRK2 channel deactivation [15]. The role of Serine 329 in the μ-opioid receptor on the potency of DAMGO, morphine, fentanyl, and β-hydroxyfentanyl was previously described [16]. In brief, this mutation demonstrated the dual role of Serine 329 in the potency of various agonists. The potency of DAMGO, a peptide agonist, decreased, whereas the potency of nonpeptide agonists, like morphine and fentanyl increased when this residue was mutated to alanine.

This experimentally created model [17] provides a defined population of functionally active opioid receptor (hMORwt, hKOR, hDOR, or hMORS329A) and an excellent tool for measuring the efficacy and potency of a ligand for a certain receptor [16,18].

2. Materials and methods

2.1. Compounds: peptide synthesis

2.1.1. Tyr-Pro-Phe-D-Pro-NH_2 *1*

This linear morphiceptin analogue was synthesized according to the Fmoc/*t*-Bu chemistry solid-phase technique. A Rink amide MBHA resin and coupling reagents HOBt/BOP were used on a Rainin PS 3 automated solid-phase peptide synthesizer. Crucial synthesis steps were monitored by means of the Kaiser test. The resin was cleaved by treatment with 95% TFA in water for 2 h. The peptide was precipitated as TFA-salt with cold diethyl ether and dried in vacuo.

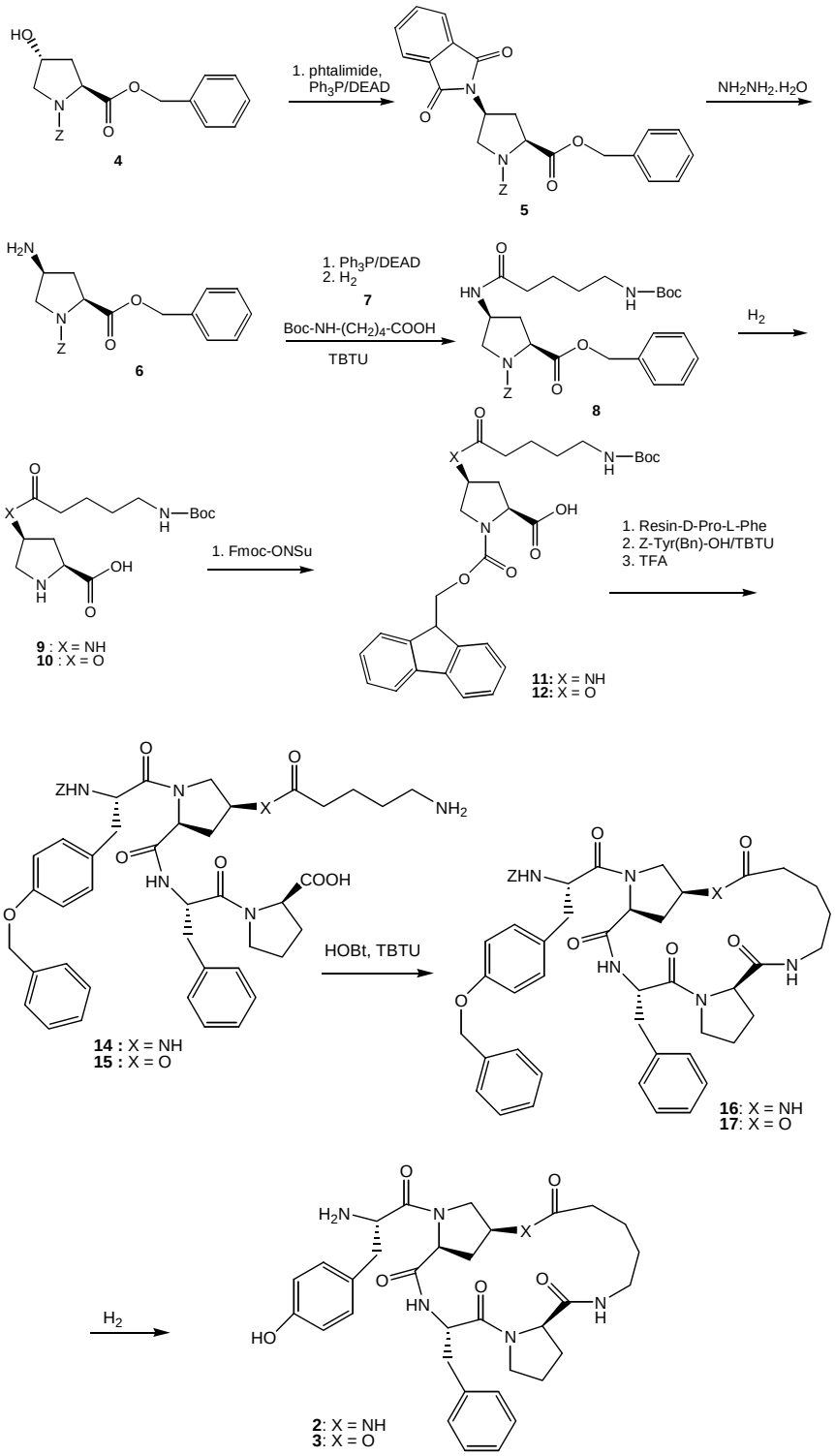

Fig. 2. Synthesis of cyclic peptides **2** and **3**.

*2.1.2. Cyclic morphiceptin analogues **2** and **3** (Fig. 2)*

*2.1.2.1. (1S,4S,10R,19S)-21-(L-Tyr)-4-benzyl-2,5,11,17-tetraoxo-3,6,12,18,21-pentaaza-tricyclo[17,2,1,$0^{6,10}$]-docosane (**2**).* *N*-*Z*-(2*S*,4*R*)-4-hydroxyproline benzyl ester was treated with phtalimide in a Mitsunobu reaction affording its *N*-phthalimido (*S*)-4-amino-derivative. The phthalimido group was cleaved with hydrazine to obtain the free amine, *N*-*Z*-(2*S*,4*S*)-4-aminoproline benzyl ester. The amino group was coupled to Boc-protected 5-aminopentanoic acid with TBTU. After removal of both *Z*- and benzylprotections with hydrogenolysis, the proline moiety was Fmoc-protected with fluoren-9-ylmethylsuccinimidyl carbonate. This protected 4-(5-aminopentanoyl)proline was a suitable building block to be used in a solid-phase synthesis procedure and was incorporated in a linear pseudopeptide with Fmoc-D-Pro, Fmoc-L-Phe, and *N*-*Z*-L-tyrosine-*O*-benzyl ether. To prevent diketopiperazine formation a 2-chlorotritylpolystyrene resin was used as polymeric support with TBTU as coupling reagent. After Boc- and resin cleavage by treatment with a 95% TFA solution in water, a linear pseudopeptide was obtained. Cyclization was achieved by coupling the *C*-terminal carboxylic acid to the amino terminus of the bridging unit, in highly diluted solution, with 3 equivalents TBTU, 3 equivalents HOBt, and 9 equivalents diisopropyletylamine as base, yielding the *Z*-and benzyl protected target compound, (1*S*,4*S*,10*R*,19*S*)-21-[*Z*(*O*)-benzyl)L-Tyr]-4-benzyl-2,5,11,17-tetraoxo-3,6,12,18,21-pentaaza-tricyclo[17,2,1,$0^{6,10}$]-docosane. Removal of the protecting groups by hydrogenolysis afforded compound **1**.

*2.1.2.2. (1S,4S,10R,19S)-21-(L-Tyr)-4-benzyl-2,5,11,17-tetraoxo-18-oxa-3,6,12,21-tetraaza-tricyclo[17,2,1,$0^{6,10}$]-docosane (8.1b) (**3**).* For the synthesis of cyclic peptide **3** in which the bridging moiety was introduced by means of an ester bond, Boc-protected 5-aminopentanoic acid, was linked to the starting *N*-*Z*-(2*S*,4*R*)-4-hydroxyproline benzyl ester under Mitsunobu conditions. The incorporation of *N*-*Z*-(2*S*,4*S*)-4-[4(*N*-Boc-aminobutyl)carbonyloxy]-proline benzyl ester in a linear pseudopeptide, the cyclization and the final deprotection of (1*S*,4*S*,10*R*,19*S*)-21-[*Z*(*O*)-benzyl)L-Tyr]-4-benzyl-2,5,11,17-tetraoxo-18-oxa-3,6,12,21-tetraaza-tricyclo[17,2,1,$0^{6,10}$]-docosane was performed as described for amide analogue **2**.

2.2. Subcloning and in vitro transcription of cDNA clones encoding GIRK1/2 channels; human μ-, κ-, and δ-opioid receptors (hMOR, hKOR, and hDOR); and RGS4

Plasmids containing the entire coding sequence for the mouse GIRK1 and the mouse GIRK2 channel were subcloned into the vector pSP35T and pBScMXT, respectively, and designated as pSP/GIRK1 [19] and pBScMXT/GIRK2 [13]. The polylinker in each of these vectors is flanked by *Xenopus* globin 5′ and 3′ untranslated regions, resulting in an enhanced protein expression after injection of in vitro transcribed cRNA [20]. For in vitro transcription, plasmids were first linearized either with *Eco*RI (for pSP/GIRK1), or with *Sal*I (for pBScMXT/GIRK2). Next the cRNAs were synthesized from the linearized plasmids using the large-scale SP6 mMessage mMachine (for pSP/GIRK1) or T3 mMessage mMachine (for pBScMXT/GIRK2) transcription kit (Ambion).

The hMOR, hKOR, hDOR, and rat RGS4 were subcloned into pGEMHE as described [17]. For in vitro transcription, each clone was linearized with *Nhe*I. Next the capped cRNA's were synthesized from the linearized plasmids using the large-scale T7 mMessage mMachine transcription kit (Ambion).

2.3. Construction of mutant human μ-opioid receptor

Ser329 in hMOR was mutated to Ala329 using the Quickchange site-directed mutagenesis kit (Stratagene). The primers were designed in such way that a silent *Mlu*I restriction site was introduced simultaneously: 5′-GCT-CTAGGTTACACAA**AC<u>GCGT</u>**GCCTCAACCCAGTCC-3′ and 5′-GGACTGGGTTGAGGC**A<u>CGCGT</u>**TTGTGT- (codon and complementary codon are underligned, palyndromic sequence is in bold). Cycling parameters were set according to the manufacturer's guidelines. The cDNAs from eight single colonies were digested with *Mlu*I to identify possible mutants. All eight clones contained the *Mlu*I restriction site, which was introduced by the mutant primers. A 313 basepair fragment containing the desired mutation was isolated by a double restriction digest with *Nsi*I and *Bgl*II. The mutant cDNA was then loaded on an agarose gel, the fragment of interest was cut out, gene-cleaned (QIAQUICK, QIAGEN) and ligated with T4 DNA ligase (Promega) into the corresponding sites of the WT hMOR/pGEMHE. The same mutant fragment was subcloned into pGEM7Zf(+) (Promega) for DNA sequencing (Eurogentec). For in vitro transcription, the mutant hMORS329A/pGEMHE was linearized with *Nhe*I. Next the capped cRNA's were synthesized from the linearized plasmids using the large-scale T7 mMessage mMachine transcription kit (Ambion).

2.4. Experimental model

Xenopus laevis oocytes were prepared for injection as described [21]. Oocytes were co-injected with 0.5 ng 50 nl^{-1} GIRK1, 0.5 ng 50 nl^{-1} GIRK2 and 10 ng 50 nl^{-1} RGS4 cRNA, with the addition of 10 ng 50 nl^{-1} of either hMOR or hMORS329A cRNA. Injected oocytes were maintained in ND-96 solution (composition in mM: KCl 2, NaCl 96, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, pH 7.5) supplemented with 50 μg ml^{-1} gentamicin sulphate and incubated at 16 °C.

2.5. Electrophysiological recordings

Whole-cell currents from oocytes were recorded one day after injection using the two-microelectrode voltage clamp technique (Geneclamp 500, Axon Instruments). Resistances of voltage and current electrodes were kept as low as possible (approx. 200 kΩ) and were filled with 3 M KCl. To eliminate the effect of voltage drop across the bath-grounding electrode, the bath potential was actively controlled. All experiments were performed at room temperature (19–23 °C). At the start and the end of each experiment, oocytes were superfused with low-potassium (ND-96) solution (composition in mM: KCl 2, NaCl 96, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, and pH 7.5). During application of increasing concentrations of ligands, oocytes were superfused with high-potassium (HK) solution (composition in mM: KCl 96, NaCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, and pH 7.5). In HK solution, the K^+ equilibrium potential is close to 0 mV and enables K^+ inward currents to flow through inwardly rectifying K^+ channels at negative holding potentials. A gravity controlled fast perfusion system (Warner Instruments) was used to ensure rapid solution exchanges. Application of opioid ligands did not evoke an increase of the conductance in uninjected oocytes ($n = 30$). In each experiment, oocytes were clamped at a holding potential of −70 mV for approximately 10 min and superfused with ND-96 solution. Next, the superfusion was switched from ND-96 to HK solution, after which increasing concentrations of an opioid receptor agonist were applied. Each concentration was applied for as long as needed to achieve a steady state GIRK1/GIRK2 current activation. Each ligand concentration was washed out by superfusing with HK solution. During this washout period, the channels return to the control current level as a result of deactivation process that is dramatically accelerated in the presence of RGS4, as described [15]. At the end of each experiment, the oocyte was superfused with HK solution containing 300 μM $BaCl_2$, causing block of the net GIRK1/GIRK2-gated inward current. Finally, the superfusion was switched back to ND-96 solution to confirm complete reversibility. To avoid that the receptor expression level affects the EC_{50}-values of the investigated agonists in the study, the expression system was standardized as previously described [17].

2.6. Data analysis

The pCLAMP program was used for data acquisition and data files (Axon Instruments) were imported in Microsoft Excel. The percentage activated current was calculated using the equation

$$\text{percentage activation} = \left(\frac{\text{activated current amplitude}}{\text{control current amplitude}} \times 100\right) - 100$$

and 0% was taken as the control current level. Current percentages were used for the calculation of the EC_{50}-value, using the Hill equation:

$$I = \frac{I_{max}}{1 + (EC_{50}/A)^{n_H}}$$

where I represents the current percentage, I_{max} the maximal current percentage, EC_{50} the concentration of the agonist that evokes the half-maximal response, A the concentration of agonist, and n_H the Hill coefficient. Averaged data are indicated as mean ± S.E.M. and were calculated using n experiments, where n indicates the number of oocytes tested. Statistical analysis of differences between groups was carried out with Student's t test and a probability of 0.05 was taken as the level of statistical significance.

Substrate activity for DPPIV was determined as described [22].

3. Results

Each receptor was individually co-expressed with GIRK1/GIRK2 channels and RGS4, mimicking the native neuronal G-protein-mediated pathway of K^+ channel activation [14]. We used the two-microelectrode voltage clamp technique to measure the opioid receptor-activated GIRK1/GIRK2 channel response as the in increase of the inward K^+ current at −70 mV, evoked by the application of increasing concentrations of opioid ligands. In our study, we examined the potency of **1** and the cyclic peptides **2** and **3** (Fig. 1) on hMORwt, the mutant receptor hMORS329A, hKOR, and hDOR.

Fig. 3 shows representative current traces of agonist-gated currents evoked from oocytes expressing hMORwt by **1** (Fig. 3A), **2** (Fig. 3B) and **3** (Fig. 3C). The agonists could not activate hKOR nor hDOR up to a concentration of 1 mM. Fig. 4 illustrates this finding with a representative current trace of an oocyte expressing hKOR upon application of 1 mM **2**.

Table 1 summarizes EC_{50} values calculated for **1**, **2**, and **3** via hMORwt and hMORS329A. Averaged data are indicated as the mean ± S.E.M. and were calculated using five to seven experiments.

Concentration-response relationships (Fig. 5A–C) are shown for **1**, **2**, and **3**, respectively.

Table 1
EC_{50} values calculated for GIRK1/GIRK2 channel activation via hMORwt or hMORS329A, co-expressed with RGS4 in *Xenopus laevis* oocytes

Receptor	EC_{50} values	
	hMORwt	hMORS329A
1	59.3 ± 4.8 nM	292.8 ± 26.9 nM
2	976.5 ± 41.7 nM	4.68 ± 0.41 μM
3	1017.7 ± 60.7 nM	4.31 ± 0.54 μM

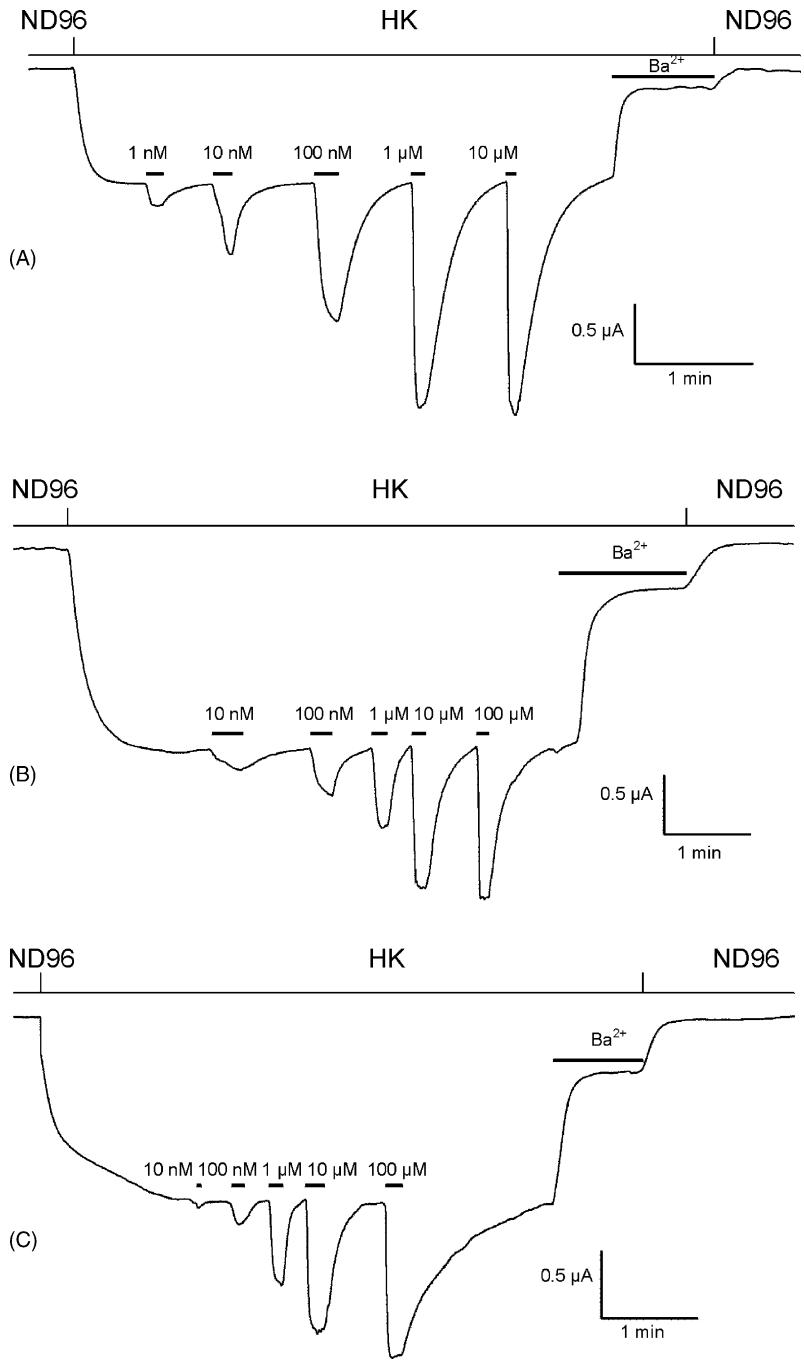

Fig. 3. Representative current traces evoked from *Xenopus laevis* oocytes co-expressing GIRK1/GIRK2 channels and RGS4 with hMORwt. Agonist-gated currents were evoked at −70 mV by application of increasing concentrations of **1** (A), **2** (B), or **3** (C). ND-96 solution contains (in mM): KCl 2, NaCl 96, $MgCl_2$ 1, $CaCl_2$ 1.8, and HEPES 5. HK solution is composed of (in mM): KCl 96, NaCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, and HEPES 5.

The linear morphiceptin analogue **1** (EC_{50} value: 59.3 ± 4.8 nM) was about 16 times more potent than the cyclic analogues via the wild-type μ-opioid receptor. Both cyclic analogues were as potent via the hMORwt (EC_{50} value 976.5 ± 41.7 nM for **2** and 1017.7 ± 60.7 nM for **3**).

On the mutant receptor, the EC_{50} values for the three agonists were four to five times higher as compared to the wild-type receptor (EC_{50} values 292.8 ± 26.9 nM for **1**, 4.68 ± 0.41 μM for **2**, and 4.31 ± 0.54 μM for **3**). Statistical analysis revealed that there was no difference in potency between **2** and **3**. **1** displayed also on the mutant hMORS329A a 16-fold higher potency than the cyclic peptides.

The cyclic proline analogues were found to be stable against hydrolysis by DPPIV. Under identical conditions the corresponding linear analogues showed half-life of about 30 min. Degradation was monitored by HPLC and a disappearance curve was constructed from the integrated peak areas versus time. To verify the specificity of the

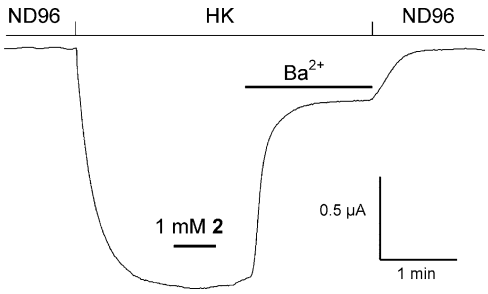

Fig. 4. Representative current trace evoked from *Xenopus laevis* oocytes co-expressing GIRK1/GIRK2 channels and RGS4 with hKOR. Currents were evoked at −70 mV by application of 1 mM of **2**.

reaction, HPLC analysis was also performed after inactivation of the enzyme by a selective irreversible inhibitor (diphenyl Pro-Prophosphonate). No peptide degradation was observed.

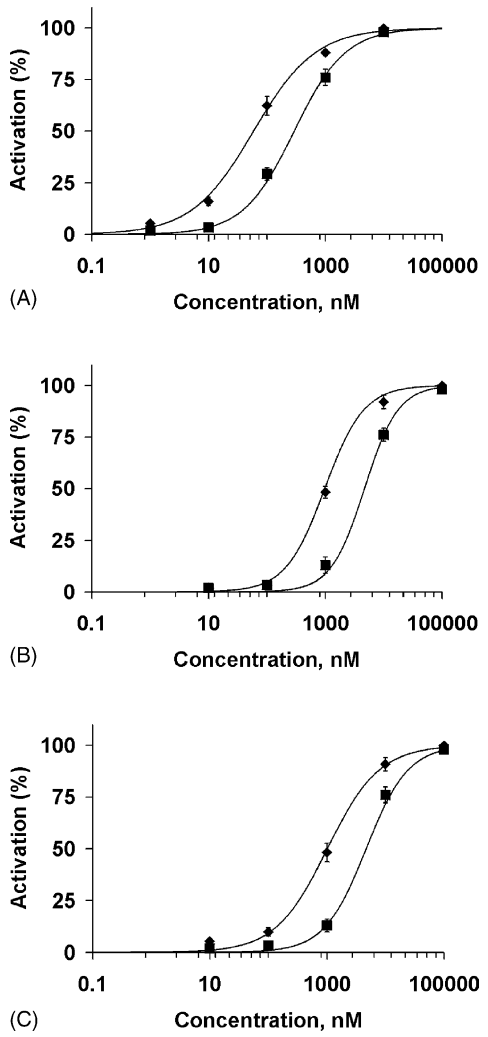

Fig. 5. Concentration–response curves for GIRK1/GIRK2 channel activation by increasing concentrations of **1** (A), **2** (B), and **3** (C). Agonist-gated currents were evoked from *Xenopus laevis* oocytes co-expressing GIRK1/GIRK2 and RGS4 with hMORwt (◆) or hMORS329A (■). The agonist-gated increase of GIRK current at each concentration was normalized to a maximal response of 100%. Each point represents the average current activation evoked from five to eight oocytes (mean ± S.E.M.).

4. Discussion

In this study, we present the synthesis and pharmacological profile of two new cyclic morphiceptin analogues. In search for biologically active and stable peptides, we designed a 6-atom bridge between the terminal amide and the Pro^2-4 position: a tetramethylene group linked to the Pro residue through an amide or ester bond. Focusing on peripheral selectivity could afford interesting therapeutic products, which would not be associated with opiate side effects linked to central opioid activity (sedation, analgesia). Such analogues need certain structural features favoring a local gastrointestinal activity: these products should be stable against breakdown by peptidases in the gastrointestinal tract, while their lipophilicity should be limited, in order to prevent absorption from the intestine and potential side effects.

The *p*-hydroxyphenethylamine (tyramine) moiety is a structural feature shared by all fused ring opiates and the *N*-terminal tyrosine of opioid peptides. The presence of this tyramine moiety is widely accepted as a requirement for high μ-affinity. ^{1}H NMR spectroscopic measurements and computer simulations were carried out on 10 stereoisomeric analogues related to morphiceptin to obtain a topochemical model that explains the bioactivity of morphiceptin [10]. The relative spatial arrangements of the pharmacophoric groups, the amine and phenolic groups of the Tyr^1 residue and the aromatic group of the Phe^3 residue were defined by a set of eight bond conformations. The torsional angles around these eight bonds required for morphiceptin bioactivity were determined. The most interesting characteristic of the model is a requirement of a *cis* amide bond linking residues Tyr^1 and Pro^2, although energetically less favored.

Recently, ^{1}H NMR spectral analysis provided evidence for the *cis* Tyr-Pro amide bond for morphiceptin and endomorphin-2, using pseudoproline-containing analogues [23]. Furthermore, it has been suggested that the fourth residue of endomorphin has less stringent stereochemical or conformational requirements. Molecular dynamics simulations and NMR data indeed show that this position is conformationally flexible and independent of the preceding three amino acids [24], the structure of residue 4 does not affect recognition at the receptor site [10].

In the search for bridging units to constrain the peptide, we used the model by Yamazaki et al. [10] to determine a possible constrained bioactive conformer. The minimum energy conformation of our analogues showed great similarity with the conformation described in this model.

On the wild-type receptor, there was no difference in EC_{50} value for both cyclic analogues: 976 ± 41.7 nM and 1017.7 ± 60.7 nM for **2** and **3**, respectively. As compared to the EC_{50} value of the linear tetrapeptide Tyr-Pro-Phe-D-Pro-NH_2 (59.3 ± 4.8 nM), the cyclic derivatives are approximately 16 times less potent. This may be due to

an unoptimized conformation. The potency of the linear peptide **1** towards the wild-type receptor is similar to the potency of fentanyl (EC_{50} value 64.9 ± 7.8 nM) and only slightly lower as compared to [D-Ala^2,NMePhe^4,Gly^5-ol]-enkephalin (DAMGO) [18]. The greater flexibility of DAMGO that may allow additional binding modes could contribute for this difference [24].

The potency of all agonists was decreased four to five times by mutating the wild-type hMOR to hMORS329A. This residue is a part of the seventh transmembrane segment of the receptor and lies deep within the binding cavity of the receptor. Recently, our group showed that mutation of this residue to alanine caused dual effects depending on the nature of the ligand [16]. We noticed a similar decrease in potency with DAMGO as we can observe here with **1**, the cyclic amide and the ester derivative, suggesting similar hydrophilic contacts deep within the binding cavity of the seventh transmembrane helix.

Interestingly, the cyclic derivates and the linear tetrapeptide are selective for the μ-opioid receptor. None of these agonists could activate GIRK1/GIRK2 channels coupled to hDOR or hKOR. Endomorphins have the highest specificity for μ receptors than any of the endogenous substances heretofore described [25]. This selectivity feature is of major importance in the quest for new therapeutic agents.

Morphiceptin is metabolized by peptidases. There is evidence that morphiceptin is primarily hydrolyzed and inactivated by dipeptidyl peptidase IV (DPP IV). Experiments with rats that were genetically deficient in DPP IV have demonstrated the role op this enzyme in the inactivation of morphiceptin [26]. The structural modifications which we introduced, as compared to the basic morphiceptin structure may provide some enzymatic resistance and make our compounds more suitable for therapeutic use. As expected, the linear compound **1** appeared a DPP IV substrate and is degraded by the enzyme. The cyclic amide analogue **2** was found to be stable against DPP IV degradation. The intramolecular bridge created by cyclization prevents the enzyme from interacting with the molecule. This may extend the half-life of the compound in vivo and favor its potential therapeutic application.

Although these cyclic peptides are designed to act locally in the gut, little is known about their possible intestinal absorption and their ability to pass the blood–brain barrier. Inconclusive results on the blood–brain barrier permeability of morphiceptin and some linear analogues were recently reported by Hau et al. [27]. Therefore, additional experiments on the absorption and brain distribution of these type of peptides are still needed.

In conclusion, the present study shows the synthesis and pharmacological characterization of two cyclic morphiceptin analogues. The 6-atom bridge constrained molecules **2** and **3** are equally potent towards the μ-opioid receptor, while no effect was observed on κ- and δ-opioid receptors. This selectivity together with the resistance to enzymatic hydrolysis provides an interesting pharmaceutical template in the promising approach for the design of potential antidiarrheal agents.

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