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Characterization of antinociceptive activity of novel endomorphin-2 and morphiceptin analogs modified in the third position

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Abstract

In the present study we investigated and compared the in vivo analgesia of centrally administered endomorphin-2 and morphiceptin, and their analogs modified in position 3. Two series of analogs were synthesized by introducing unnatural aromatic amino acids in the D configuration: 3-(1-naphthyl)-D-alanine (D-1-Nal), 3-(2-naphthyl)-D-alanine (D-2-Nal), 3-(4-chlorophenyl)-D-alanine (D-ClPhe), 3-(3,4-dichlorophenyl)-D-alanine (D-Cl_2Phe). Antinociceptive activity of endomorphin-2, morphiceptin, and their analogs was compared in the mouse hot-plate test, performed after i.c.v. administration of the peptides at a dose of 10 μg/animal. The best results were obtained for two morphiceptin analogs, [D-Phe^3]morphiceptin and [D-1-Nal^3]morphiceptin, which showed greatly improved analgesic activity, as compared to morphiceptin. In the endomorphin-2 series none of the modifications produced analogs more potent than the parent compound, but [D-1-Nal^3]endomorphin-2 was the best analog. Antinociception induced by endomorphin-2 was reversed by concomitant i.c.v. administration of [D-Phe^3]endomorphin-2, [D-2-Nal^3]endomorphin-2, and [D-2-Nal^3]morphiceptin, indicating that these analogs were weak μ-opioid antagonists.

Keywords: μ-Opioid receptor; Hot-plate test; Peptide analogs; Agonists; Antagonists; Naloxone

1. Introduction

Opioids, including morphine, and other plant alkaloids rank as the most effective analgesics available today [1]. Pain relief effects are mediated by three main receptor types (μ, δ, and κ), which were postulated about 25 years ago. The search for endogenous opioids that activate μ-receptor, mediating the most potent antinociceptive effects [2], led quite recently to the discovery of two tetrapeptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2). Endomorphins exhibit the highest affinity and selectivity for the μ-receptor of any compounds found so far in the mammalian nervous system [3]. The specific action of endomorphin-1 and -2 in stimulating μ-opioid receptors in vitro is consistent with in vivo antinociceptive activity [4–7].

A number of data are available as concerns the antinociceptive effects of endomorphin-1 and/or -2 after intracerebroventricular (i.c.v.) [3,4,8,9], intrathecal (i.t.) [3,4,6,10,11] or peripheral administration in awake mice or rats in acute heat (tail-flick or hot-plate) or mechanical pain tests. In all cases endomorphins exhibited potent antinociceptive effect, although the potencies of the peptides and the duration of the effects depended on the species, on the pain test applied and on the route of administration. Similar potencies of the endomorphins were found in the tail-flick and the hot-plate tests after i.c.v. administration in some studies [12,13], whereas Tseng et al. [7] found a higher potency in the hot-plate test than in the tail-flick test.

Endomorphins are in the same class of peptides as morphiceptin (Tyr-Pro-Phe-Pro-NH_2) [14], in which Pro at the second position confers high selectivity on the μ-opioid receptor. The structure of morphiceptin differs from endomorphin-2 only by the amino acid in the fourth position (Pro and Phe, respectively). Endomorphins and

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morphiceptin have a unique N-terminal Tyr-Pro sequence different from the N-terminal tetrapeptide sequence Tyr-Gly-Gly-Pro, characteristic for typical opioids, like enkephalins, endorphin and dynorphins [15]. Despite this different amino acid sequence, endomorphins and morphiceptin produce similar physiological effects to the traditional endogenous opioids and elicit equipotent analgesia to morphine [3].

Phenylalanine in position 3 of endomorphin-2 and morphiceptin analogs plays a key role in the interaction of these peptides with opioid receptors. Topographical location of the aromatic ring of the position 3 amino acid residue, as well as its lipophilicity, seems to be critical for the μ-receptor binding [16].

In the present study we investigated and compared the in vivo analgesia of centrally administered endomorphin-2 and morphiceptin and their analogs modified in position 3 by introducing different unnatural amino acids.

2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesized by standard solid-phase procedures as described before [17], using techniques for Boc-protected amino acids on *p*-methylbenzhydrylamine (MBHA) resin (100–200 mesh, 0.8 mM/g, Novabiochem). Fifty percent trifluoroacetic acid (TFA) in dichloromethane was used for deprotection of Boc-groups and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was employed to facilitate coupling. Simultaneous deprotection and cleavage from the resin was accomplished by treatment with 90% anhydrous hydrogen fluoride (HF) and 10% anisole scavenger at 0 °C for 1 h. Crude peptides were purified by RP HPLC on a Vydac C_{18} column (1 cm × 25 cm) using the solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient. Calculated values for protonated molecular ions were in agreement with those obtained using FAB-mass spectrometry. The physicochemical data of the analogs are summarized in Table 1.

2.2. Opioid receptor binding assays

Receptor binding assay was performed as described previously [18]. Crude membrane preparations, isolated from Wistar rat brains, were incubated at 25 °C for 120 min with 0.5 nM [^{3}H]naloxone in a total volume of 1 ml of 50 mM Tris–HCl (pH 7.4) containing bovine serum albumin (BSA) (1 mg/ml), bacitracin (50 μg/ml), bestatin (30 μM) and captopril (10 μM). All reactions were carried out in duplicate, at 10 μM peptide concentration. Incubations were terminated by rapid filtration through GF/B Whatman glass fiber strips, using Brandel 24 Sample Semi-Auto Harvester. The filters were washed with 4 ml of ice-cold saline solution and the bound radioactivity was measured in the liquid scintillation counter L55000 TA (Beckman). Nonspecific binding was determined in the presence of naltrexone hydrochloride (10 mM). The data were analyzed by a nonlinear least square regression analysis computer program Prism Graph Pad.

2.3. Antinociception

The procedures used in this study were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and approved by the Local Ethical Committee for Animal Research with the following numbers: N/10-04-04-12 and N/12-04-04-14.

Male Swiss albino mice (CD1, Charles River), weighing 20–22 g, were used throughout the study. The animals were housed 30 per Makrolon box (L: 40, W: 25, H: 18 cm), with free access to standard semi-synthetic laboratory diet and tap water ad libitum, under controlled environmental conditions (temperature: 22 ± 1 °C, 7 a.m.–7 p.m. light–dark cycle). Mice were tested only once and sacrificed immediately thereafter by decapitation. To assess the antinociceptive effects of the opioids, the hot-plate test was used.

Intracerebroventricular (i.c.v.) injections (10 μl) were performed in the left brain ventricle of manually immobilized mice with a Hamilton microsyringe (50 μl) connected to a needle (diameter 0.5 mm), as described by Haley and Mc Cormick [19]. All drugs for i.c.v. administration were dissolved in 0.9% NaCl.

Table 1
Physicochemical data of new endomorphin-2 analogs

Peptide no.	Sequence	HPLC[a] (t_R)	FAB-MS		
			Formula	MW	$[M + H]^+$
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	12.51	$C_{32}H_{37}N_5O_5$	571	572
2	Tyr-Pro-D-Phe-Phe-NH_2	13.50	$C_{32}H_{37}N_5O_5$	571	572
3	Tyr-Pro-D-1-Nal-Phe-NH_2	16.05	$C_{36}H_{39}N_5O_5$	621	622
4	Tyr-Pro-D-2-Nal-Phe-NH_2	16.15	$C_{36}H_{39}N_5O_5$	621	622
5	Tyr-Pro-D-ClPhe-Phe-NH_2	15.53	$C_{32}H_{36}ClN_5O_5$	605	606
6	Tyr-Pro-D-Cl_2Phe-Phe-NH_2	16.64	$C_{32}H_{35}Cl_2N_5O_5$	639	640

[a] HPLC elution on a Vydac C_{18} column (1 cm × 25 cm) using the solvent system of 0.1% TFA in water/80% acetonitrile in water containing 0.1% TFA and a linear gradient of 20–90% B over 25 min.

The hot-plate test was performed according to the method of Eddy and Leimbach [20]. A transparent plastic cylinder (14 cm diameter; 20 cm height) was used to confine the mouse on the heated (55 ± 0.5 °C) surface of the plate. The animals were placed on the hot-plate 5 min after i.c.v. injection of saline or peptides and the latencies to paw licking, rearing and jumping were measured. A cut-off time of 240 s was used to avoid tissue injury.

The data are expressed as mean ± S.E.M. Differences between groups were assessed by an analysis of variance (ANOVA). Antagonist effects of peptides in the combination experiments were analyzed using two-way analysis of variance (ANOVA) and a post hoc multiple comparison Student–Newman–Keuls test was used for multiple comparisons between groups. A probability level of 0.05 or smaller was used to indicate statistical significance.

To evaluate the hot-plate test responses detailed below, the control latencies (t_0) and test latencies (t_1) were determined after injection of saline and a peptide, respectively. The percentage of maximal possible effect (%MPE) was calculated as $\%MPE = (t_1–t_0)/(t_2–t_0) \times 100$, where the cut-off time (t_2) was 240 s.

The median antinociceptive dose (ED_{50}) was calculated according to the method of Litchfield and Wilcox [21].

3. Results

Endomorphin-2 and morphiceptin are parent compounds of two series of analogs modified in position 3 by introducing unnatural aromatic amino acids in the D configuration: 3-(1-naphthyl)-D-alanine (D-1-Nal), 3-(2-naphthyl)-D-alanine (D-2-Nal), 3-(4-chlorophenyl)-D-alanine (D-ClPhe), 3-(3,4-dichlorophenyl)-D-alanine (D-Cl_2Phe). μ-Receptor binding affinities for both series, as measured by IC_{50} values against [^{3}H]naloxone, are provided in Table 2. Introduction of D-1-Nal residue in position 3 produced the most potent analogs in both, endomorphin-2 and morphiceptin series (89.1 ± 3.8 nM and 1.9 ± 0.2 nM, respectively). Antinociceptive activity of endomorphin-2, morphiceptin, and their analogs was compared in the mouse hot-plate test, performed after i.c.v. administration of the peptides at a dose of 10 μg/animal. Latencies expressed as %MPE to paw licking, rearing, and jumping, are summarized in Table 3. The best results were obtained for two morphiceptin analogs, [D-Phe^3]morphiceptin and [D-1-Nal^3]morphiceptin, which showed greatly improved analgesic activity, as compared to morphiceptin. In the endomorphin-2 series none of the modifications produced analogs more potent than the parent compound, but [D-1-Nal^3]endomorphin-2 (**3**) was the best analog, showing that D-1-Nal^3 modification is the most favorable in both series of peptides, which is in good agreement with the binding data. Additionally, naloxone (5 μg, i.c.v.) was used to confirm that the action of new endomorphin-2 analogs was mediated through the μ-opioid receptors. As shown in Fig. 1, the analgesic effect of [D-1-Nal^3]endomorphin-2 (**3**) was antagonized by concomitant administration of naloxone.

Table 2
μ-Opioid receptor binding affinities of endomorphin-2 and morphiceptin analogs

Peptide no.	Sequence	IC_{50} ± S.E.M. (nM)
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	3.9 ± 0.2
2	Tyr-Pro-D-Phe-Phe-NH_2	310.1 ± 13.5
3	Tyr-Pro-D-1-Nal-Phe-NH_2	89.1 ± 3.8
4	Tyr-Pro-D-2-Nal-Phe-NH_2	>1000
5	Tyr-Pro-D-ClPhe-Phe-NH_2	>1000
6	Tyr-Pro-D-Cl_2Phe-Phe-NH_2	891.0 ± 45.0
7	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)[a]	79.4 ± 3.4
8	Tyr-Pro-D-Phe-Pro-NH_2	50.1 ± 3.1
9	Tyr-Pro-D-1-Nal-Pro-NH_2	1.9 ± 0.2
10	Tyr-Pro-D-2-Nal-Pro-NH_2	158.5 ± 11.5
11	Tyr-Pro-D-ClPhe-Pro-NH_2	>1000
12	Tyr-Pro-D-Cl_2Phe-Pro-NH_2	20.0 ± 2.8

[a] Data for morphiceptin analogs are from reference [18].

Table 3
Antinociceptive effect of endomorphin-2 and morphiceptin analogs in the mouse hot-plate test after i.c.v. injection (10 μg)

Peptide no.	Sequence	Latencies (%MPE) to					
		Paw licking	Relative potency	Rearing	Relative potency	Jumping	Relative potency
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	15.25 ± 4.51	1	27.35 ± 4.29	1	72.11 ± 5.62	1
2	Tyr-Pro-D-Phe-Phe-NH_2	0.68 ± 0.22	0.04	0.75 ± 0.66	0.03	0.82 ± 0.43	0.01
3	Tyr-Pro-D-1-Nal-Phe-NH_2	6.88 ± 1.21	0.45	15.60 ± 3.37	0.57	40.12 ± 6.06	0.56
4	Tyr-Pro-D-2-Nal-Phe-NH_2	0.85 ± 0.39	0.06	1.59 ± 0.97	0.06	0.22 ± 0.08	<0.01
5	Tyr-Pro-D-ClPhe-Phe-NH_2	0.64 ± 0.28	0.04	0.31 ± 0.07	0.01	0.98 ± 0.66	0.14
6	Tyr-Pro-D-Cl_2Phe-Phe-NH_2	0.77 ± 0.29	0.05	0.66 ± 0.05	0.02	3.22 ± 0.98	0.04
7	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)[a]	11.52 ± 4.19	0.76	33.08 ± 6.54	1.21	75.12 ± 8.48	1.04
8	Tyr-Pro-D-Phe-Pro-NH_2	43.36 ± 10.27	2.84	65.49 ± 8.39	2.39	88.85 ± 4.52	1.23
9	Tyr-Pro-D-1-Nal-Pro-NH_2	44.48 ± 8.63	2.92	75.84 ± 6.63	2.77	92.72 ± 5.57	1.29
10	Tyr-Pro-D-2-Nal-Pro-NH_2	0.64 ± 0.33	0.04	1.79 ± 0.90	0.07	8.22 ± 5.36	0.11
11	Tyr-Pro-D-ClPhe-Pro-NH_2	3.64 ± 0.96	0.24	13.36 ± 5.64	0.49	32.28 ± 10.25	0.45
12	Tyr-Pro-D-Cl_2Phe-Phe-NH_2	3.64 ± 0.71	0.24	8.92 ± 2.52	0.33	48.36 ± 7.61	0.67

[a] Data for morphiceptin analogs from reference [18].

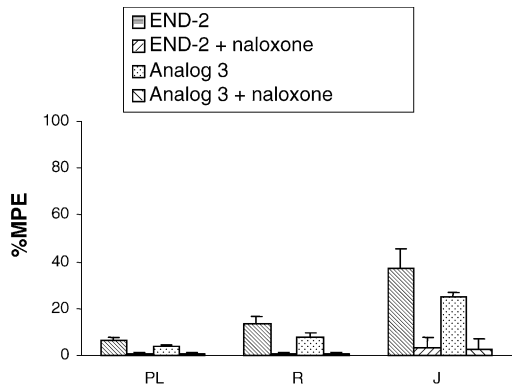

Fig. 1. Comparison of antagonistic properties of naloxone on endomorphin-2 (END-2) and [D-1-Nal^3]endomorphin-2 (Analog **3**) induced analgesia. PL, paw licking; R, rearing; J, jumping.

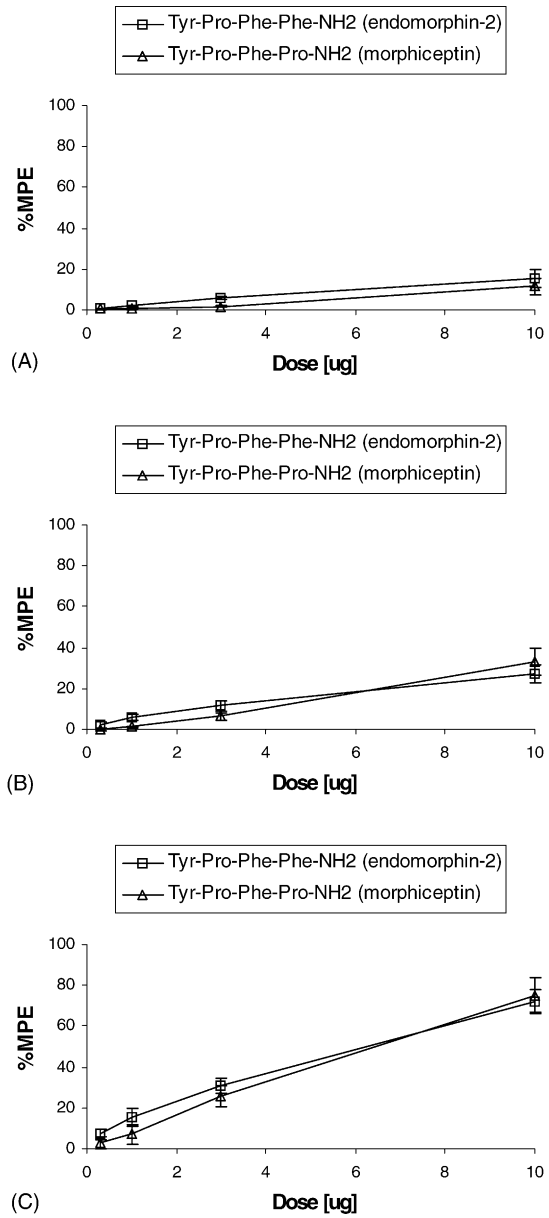

Fig. 2. Dose–response curves for the hot-plate inhibition of paw licking (A), rearing (B), and jumping (C) induced by i.c.v. injection of endomorphin-2 and morphiceptin.

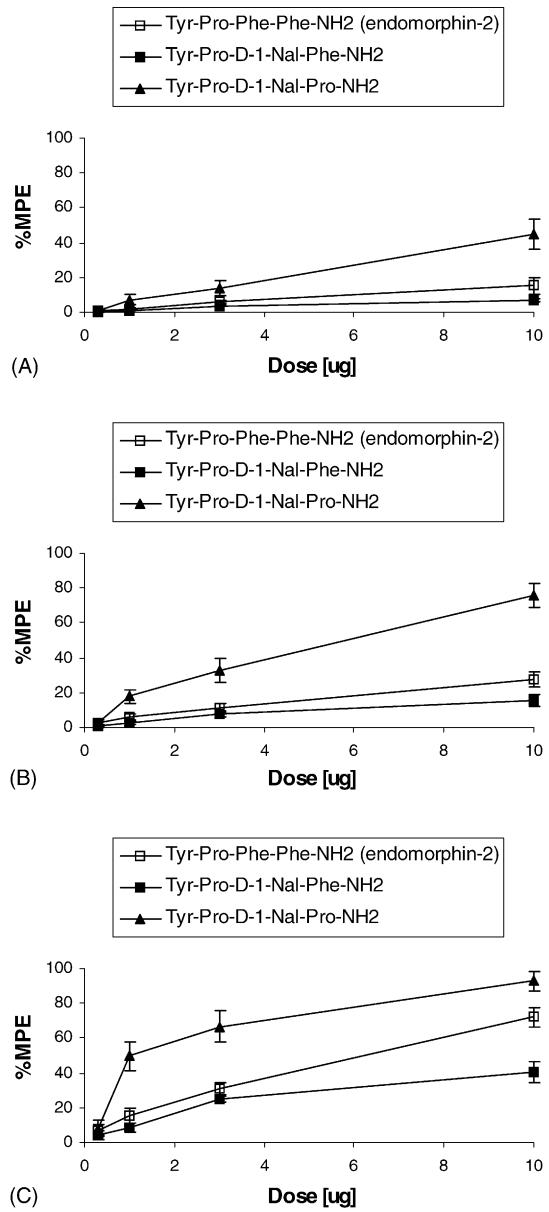

Fig. 3. Dose–response curves for the hot-plate inhibition of paw licking (A), rearing (B), and jumping (C) induced by i.c.v. injection of endomorphin-2 and [D-1-Nal^3]analogs.

Analgesic effects of endomorphin-2 and morphiceptin were both dose–dependent and were compared in Fig. 2. No significant differences between the action of these two ligands were found under these experimental conditions. At the highest dose tested (10 μg) %MPE for endomorphin-2 were 15.25, 27.35, and 72.11 and for morphiceptin were 11.52, 33.08, and 75.12 for paw licking, rearing, and jumping, respectively.

In Fig. 3 dose–response curves for endomorphin-2 and the best analog in each series, which were [D-1-Nal^3]endomorphin-2 and [D-1-Nal^3]morphiceptin, were compared. [D-1-Nal^3]morphiceptin showed the most pronounced analgesic effect, which was much higher than that of endomorphin-2. The effect produced by [D-1-Nal^3]endo-

Table 4
ED_{50} values for endomorphin-2, morphiceptin and the most potent analogs

Peptide no.	Sequence	ED_{50} (μg)		
		Paw licking	Rearing	Jumping
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	>1000	946.45	1.83
3	Tyr-Pro-D-1-Nal-Phe-NH_2	>1000	>1000	8.32
7	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)[a]	>1000	378.08	1.75
9	Tyr-Pro-D-1-Nal-Pro-NH_2[a]	53.57	3.55	0.50

[a] Data from reference [18].

Table 5
Determination of antagonistic properties of selected endomorphin-2 and morphiceptin analogs in the mouse hot-plate test after i.c.v. injection

Compounds injected	Latencies (%MPE) to		
	Paw licking	Rearing	Jumping
Endomorphin-2	6.22 ± 1.23	13.42 ± 3.04	36.97 ± 8.40
Endomorphin-2 + Tyr-Pro-D-Phe-Phe-NH_2 (**2**[a])	4.07 ± 0.76	7.76 ± 1.49	18.51 ± 2.99
Endomorphin-2 + Tyr-Pro-D-2-Nal-Phe-NH_2 (**4**[a])	3.43 ± 0.60	7.81 ± 1.12	15.96 ± 2.42
Endomorphin-2 + Tyr-Pro-D-ClPhe-Phe-NH_2 (**5**[a])	6.34 ± 1.06	14.67 ± 2.33	42.46 ± 6.69
Endomorphin-2 + Tyr-Pro-D-Cl_2Phe-Phe-NH_2 (**6**[a])	7.20 ± 0.98	13.65 ± 2.78	44.84 ± 9.14
Endomorphin-2 + Tyr-Pro-D-2-Nal-Pro-NH_2 (**10**[a])	4.24 ± 0.57	9.63 ± 1.13	20.01 ± 3.52
Endomorphin-2 + naloxone[b]	0.94 ± 0.47	0.89 ± 0.47	3.33 ± 4.58

[a] Endomorphin-2 and an analog were injected at the dose of 3 and 10 μg, respectively.
[b] Endomorphin-2 and naloxone were injected at the dose of 3 and 5 μg, respectively.

morphin-2 was also dose–dependent, but %MPE values for each dose were lower than those obtained for endomorphin-2. The ED_{50} values for endomorphin-2, morphiceptin, **3**, and **9** are shown in Table 4.

Analogs that did not show any analgesic activity were tested for their possible antagonistic properties (Table 5). Antinociception induced by endomorphin-2 was reversed by concomitant i.c.v. administration of [D-Phe^3]endomorphin-2 (**2**), [D-2-Nal^3]endomorphin-2 (**4**), and [D-2-Nal^3]-morphiceptin (**10**), indicating that these analogs were μ-opioid antagonists. In Fig. 4 the antagonistic action of analogs **2**, **4**, and **10** was compared with naloxone (5 μg) after concomitant i.c.v. administration. The order of antagonistic potency was naloxone ≫ **4** > **2** > **10**.

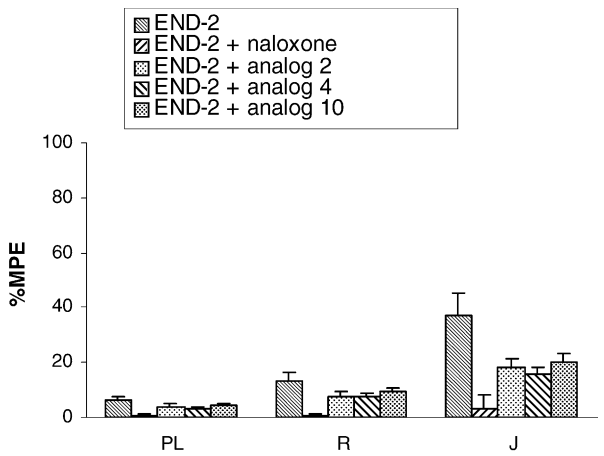

Fig. 4. Comparison of antagonistic properties of naloxone and position 3-modified endomorphin-2 and morphiceptin analogs on endomorphin-2 (END-2) induced analgesia. PL, paw licking; R, rearing; J, jumping.

4. Discussion

Endomorphin-2 and morphiceptin are selective ligands of the μ-opioid receptor, but endomorphin-2 was found to have much higher affinity for this receptor than morphiceptin [3,22]. Analgesic effects of these two peptides after i.c.v. and i.v. administration were compared by Hau et al. [23] in the tail-flick test. An equimolar dose of endomorphin-2 exhibited a significantly increased analgesic profile compared to morphiceptin. In contrast, i.v. administration of endomorphin-2 showed a significantly decreased antinociceptive activity compared to morphiceptin.

In our experiments both peptides, endomorphin-2 and morphiceptin, showed similar analgesic profile in the hot-plate test after i.c.v. administration. Nociceptive pathways activated in the hot-plate and tail-flick tests are not the same [24]. Therefore, in different antinociceptive tests the same opioid ligand can elicit different responses, even though the same receptors are involved.

Among numerous μ-opioid selective analogs synthesized so far, not many have been found to possess any antagonistic properties. Alkaloid antagonists, such as naloxone, are still the most commonly used in the μ-receptor studies, despite their limited selectivity. The first peptide antagonists with high μ-receptor selectivity were discovered through modifications of somatostatin [25,26]. Sasaki and Chiba [27] have described deltorphin (Tyr-D-MetPhe-His-Leu-Met-Asp-

NH_2, DLT) analogs, containing *N*-substituted glycine and demonstrated that such modifications produce significant differences in biological responses, including antagonist activities. For example, [N^{α}-isobutyl-Gly^6]DLT behaved as a mixed μ antagonist/δ agonist. In subsequent studies, some novel μ-opioid antagonist were obtained by substituting, *N*,*N*-bis(*p*-hydroxybenzyl)Gly residue for Tyr^1 in the μ selective dermorphin analog Tyr-D-Arg-Phe-β-Ala-NH_2 [28]. In a different approach 2′,6′-dimethyl substitution of the Tyr^1 residue of opioid agonist peptides and deletion of the positively charged N-terminal amino group or its replacement with a methyl group has been shown to represent a general structural modification to convert opioid peptide agonists into antagonists [29]. Following this concept Schiller et al. [30] synthesized the Dhp^1-analog [Dhp = 3-(2,6-dimethyl-4-hydroxyphenyl)propionic acid] of endomorphin-2, which however showed relatively low μ antagonist activity.

In the present study it was shown that some position 3 modifications of μ selective peptides, endomorphin-2 and morphiceptin, can also produce antagonism. In case of endomorphin-2, antagonistic properties were found for D-Phe^3 and D-2-Nal^3 substituted analogs. In the morphiceptin series, D-2-Nal^3 substituted analog was also a weak antagonist, while [D-Phe^3]morphiceptin was an even more potent agonist than morphiceptin. D-1-Nal^3 modification in both peptides, endomorphin-2 and morphiceptin, produced agonist peptides.

The presented results support earlier data [31] that the C-terminal residues play a critical role in the biological activity of μ specific ligands. In our studies of endomorphin-2 and morphiceptin (Phe or Pro as the C-terminal residue, respectively) substitution in the third position with the same amino acid resulted in a significant change in antinociceptive activity or a completely different mode of action, producing either agonists or antagonists.

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