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Synthesis and biological activity of N-methylated analogs of endomorphin-2

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Abstract—In this paper, we describe the synthesis of a series of endomorphin-2 analogs containing N-methylated amino acids, consecutively in each position. The μ-opioid receptor binding affinities of the new analogs were determined in the displacement experiments. Their in vivo antinociceptive activity was assessed in the hot-plate test in mice after central (icv) and peripheral (ip) administration. [Sar^2]endomorphin-2, which had the highest μ-receptor affinity, also showed the strongest analgesic effect when administered centrally and was the only analog that retained activity after peripheral injection.

1. Introduction

Centrally acting opiates, such as morphine, are the most frequently used analgesics for the relief of severe pain, though they are known to bring about a number of well-known side-effects, including tolerance, physical dependence, respiratory depression, and adverse gastrointestinal effects.[1,2] It is well-established that morphine and related alkaloids elicit their analgesic effects when bound to the μ-opioid receptor.[3] Even though δ- and κ-selective endogenous opioid peptides were discovered almost three decades ago,[4,5] no endogenous μ-selective opioids have been known until recently. Two opioid peptides, endomorphin-1 and endomorphin-2, isolated in 1997 first from bovine[6] and later from human brain cortex[7] revealed high affinity for the μ-opioid receptor.[8] These peptides have been shown to displace [^{3}H]DAMGO in the binding assay and inhibit electrically stimulated contractions of guinea pig ileum, which is consistent with the action of μ-opioid receptor agonists.[9] In behavioral experiments, intracerebroventricular (icv) or intrathecal (it) administration of endomorphin-1 and -2 produces potent analgesia which is blocked by concomitant treatment with a nonspecific opioid antagonist, naloxone, or a selective μ-opioid receptor antagonist, β-funaltrexamine.[10] In the μ-opioid receptor knock-out mice, neither endomorphin-1 nor -2 produces any significant antinociceptive effects.[11] These findings strongly indicate that the μ-opioid receptor plays an essential role in mediating endomorphin-1 and -2-induced antinociception.

Strong analgesic activity of endomorphins opens up the possibility of developing a novel class of painkillers based on their structure. Unfortunately, exogenous application of native opioid peptides is generally not successful, on account of their biological instability. Endomorphins are easily degraded by peptidases. Stone et al.[12] observed short lasting antinociceptive effects of these peptides. Spetea et al.[13] described the binding characteristics of [^{3}H]endomorphin-2 in a rat brain membrane preparation and used peptidase inhibitors in a radioligand binding assay. In the absence of pepti-

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dase inhibitors, 40% of the radioligand gets destroyed after incubation for 45 min at 25 °C.

The cerebrospinal fluid and central nervous system contain amino-, endo-, and carboxypeptidases capable of degrading opioid peptides.[14] The stability of native peptides can be increased by introducing different structural modifications into their structure. One approach is to synthesize analogs containing D-amino acids. A series of endomorphin-1 and endomorphin-2 analogs containing enantiomers of all amino acids present in their sequence has been synthesized. These analogs, however, exhibited only poor affinities toward μ-opioid receptors.[15,16] The results obtained by Cardillo et al.[17] who introduced β-amino acids into the sequence of endomorphin-1, have indicated that the presence of β-proline in position 2 is sufficient to confer good resistance against hydrolysis of a biologically important Pro-Trp bond. In the present study, we synthesized a series of endomorphin-2 analogs, containing N-methylated amino acids, to study the effect of these structural changes on the binding affinity at the μ-opioid receptor and the in vivo antinociceptive activity in mice in the hot-plate test after icv and ip administration.

2. Results

2.1. Structure–activity relationships

Systematic investigation of the effect of N-methylation of all amino acid residues in the structure of endomorphin-2 was performed in this study. [NMeTyr^1]endomorphin-2, [Sar^2]endomorphin-2, [NMePhe^3]endomorphin-2, and [NMePhe^4]endomorphin-2 were synthesized using standard solid-phase procedures and Fmoc-protected amino acids. The physicochemical data of the analogs are given in Table 1.

The ability of the obtained peptides to bind to the μ-opioid receptor was assessed using [^{3}H]naloxone in competitive radioligand binding experiments and their equilibrium inhibition constants (IC_{50}) were determined. The binding affinity of the new analogs was compared to that of endomorphin-2 and the data are given in Table 2. Substitution of NMeTyr in position 1 and NMePhe in position 3 (analogs **2** and **4**, respectively) produced about a 4-fold decrease in IC_{50} values compared to the parent compound (8.91 ± 1.23 and 8.13 ± 0.56, respectively, compared to those of 1.95 ± 0.46 nM for endomorphin-2). Analog **3** with Sar (NMeGly) in position 2 was almost equipotent with endomorphin-2 (IC_{50} 2.29 ± 0.51 nM). Analog **5** containing NMePhe in position 4 was 40-fold less potent than endomorphin-2 (IC_{50} 77.6 ± 6.65 nM).

2.2. Antinociceptive activity

It has been proposed that the hot-plate analgesia in mice is mainly due to the activation of supraspinal μ-opioid receptors, whereas tail flick analgesia at the spinal levels appears to mainly involve δ-opioid receptor activation.[23,24] In our experiments, antinociceptive activity was assessed in the hot-plate test after icv and ip injection of the peptides.

The results of the hot-plate test after icv administration of endomorphin-2 analogs are shown in Table 3. Groups of mice were injected icv with 10 μg of analogs and the hot-plate responses were measured 5 min after injection. In our earlier studies, we have established that after this time period the antinociceptive effect was most pronounced.[18] %MPE obtained for the N-methylated analogs were compared with the data for endomorphin-2. Analog **4** was equipotent, while analogs **2** and **3** were slightly better than endomorphin-2. The least potent analog **5** showed only about 1% of endomorphin-2

Table 1. Physicochemical data of endomorphin-2 N-methylated analogs

Peptide No.	Sequence	HPLC[a] (t_r)	FAB-MS		
			Formula	MW	$[M+H]^+$
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	16.21	$C_{32}H_{37}N_5O_5$	571	572
2	NMeTyr-Pro-Phe-Phe-NH_2	16.74	$C_{33}H_{39}N_5O_5$	585	586
3	Tyr-Sar-Phe-Phe-NH_2	16.31	$C_{31}H_{36}N_5O_5$	558	559
4	Tyr-Pro-NMePhe-Phe-NH_2	17.20	$C_{33}H_{39}N_5O_5$	585	586
5	Tyr-Pro-Phe-NMePhe-NH_2	21.28	$C_{33}H_{39}N_5O_5$	585	586

[a] HPLC elution on a Vydac C_{18} column (1 cm × 25 cm) using the solvent system of 0.1% TFA in water (A) and 80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 20–70% B over 30 min.

Table 2. Opioid receptor binding data of endomorphin-2 N-methylated analogs

Peptide No.	Sequence	IC_{50} ± SEM (nM)	
		μ[a]	Relative potency
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	1.95 ± 0.46	1.00
2	NMeTyr-Pro-Phe-Phe-NH_2	8.91 ± 1.23	0.22
3	Tyr-Sar-Phe-Phe-NH_2	2.29 ± 0.51	0.85
4	Tyr-Pro-NMePhe-Phe-NH_2	8.13 ± 0.56	0.24
5	Tyr-Pro-Phe-NMePhe-NH_2	77.6 ± 6.65	0.03

[a] Displacement of [^{3}H]naloxone.

Table 3. The effects of endomorphin-2 N-methylated analogs in the mouse hot plate test ($n = 10$)

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)	13.73 ± 2.27	1.00	24.78 ± 3.04	1.00	64.68 ± 8.03	1.00
2	NMeTyr-Pro-Phe-Phe-NH_2	16.57 ± 2.86	1.21	30.16 ± 3.54	1.22	77.64 ± 7.44	1.20
3	Tyr-Sar-Phe-Phe-NH_2	18.04 ± 4.26	1.31	35.20 ± 3.88	1.42	67.78 ± 7.99	1.05
4	Tyr-Pro-NMePhe-Phe-NH_2	12.13 ± 2.19	0.88	23.18 ± 3.93	0.94	61.54 ± 9.32	0.95
5	Tyr-Pro-Phe-NMePhe-NH_2	0.88 ± 0.19	<0.01	1.24 ± 0.31	<0.01	1.85 ± 0.35	<0.01

activity (Table 3). For endomorphin-2 and analogs **2**, **3**, and **4**, dose–response curves were obtained (Fig. 1).

The results of the hot-plate test after ip administration of endomorphin-2 analogs are shown in Figure 2. In contrast to the analgesic profile seen with icv administration, ip injection of endomorphin-2 did not produce any significant analgesic activity. Among the N-methylated analogs, only **3** showed significant antinociceptive activity.

3. Discussion

The majority of opioid peptides undergo rapid enzymatic degradation, particularly in blood, by exo- and endopeptidases. Due to this reason and due to their low permeation across the blood–brain barrier, they cannot reach the central nervous system using up an amount sufficient to elicit analgesia following peripheral administration.[24] Endomorphins appear to be vulnerable to rapid degradation by peptidases.[25] Stabilization against peptidases in vivo leads to prolonging the half-life, which may allow the peptide to reach the brain. It has been shown that small peptides can slowly cross the blood–brain barrier by simple diffusion or by saturable transport systems.[26] Recently, Hau et al.[27] have reported that cationization of endomorphin-2 by guanidine addition increased the peptide's half-life, blood–brain barrier transport, and analgesic profile. Therefore, appropriate structural modifications to endomorphins may modify their pharmacological properties following peripheral administration. For example, an analog of endomorphin-1, Tyr-β-Pro-Trp-Phe-NH_2, was reported to be an effective antinociceptive agent in the tail-flick test after sc injection in mice.[28] This analog is among the first endomorphin-1 analogs showing antinociceptive activity after systemic administration.

In continuance of our structure–activity relationship studies of μ-selective opioid peptides, we have synthesized a series of N-methylated analogs of endomorphin-2. In each analog, one amino acid in the sequence of endomorphin-2 was changed. Tyrosine in position 1 and phenylalanine in positions 3 and 4 were replaced by their N-methylated equivalents, while N-methylglycine (sarcosine) was substituted for proline. The highest μ-receptor binding affinity, almost equal to that of the native endomorphin-2, was found for [Sar^2]endomorphin-2, while [$NMePhe^4$]endomorphin-2 had the lowest μ-affinity. The binding data were in

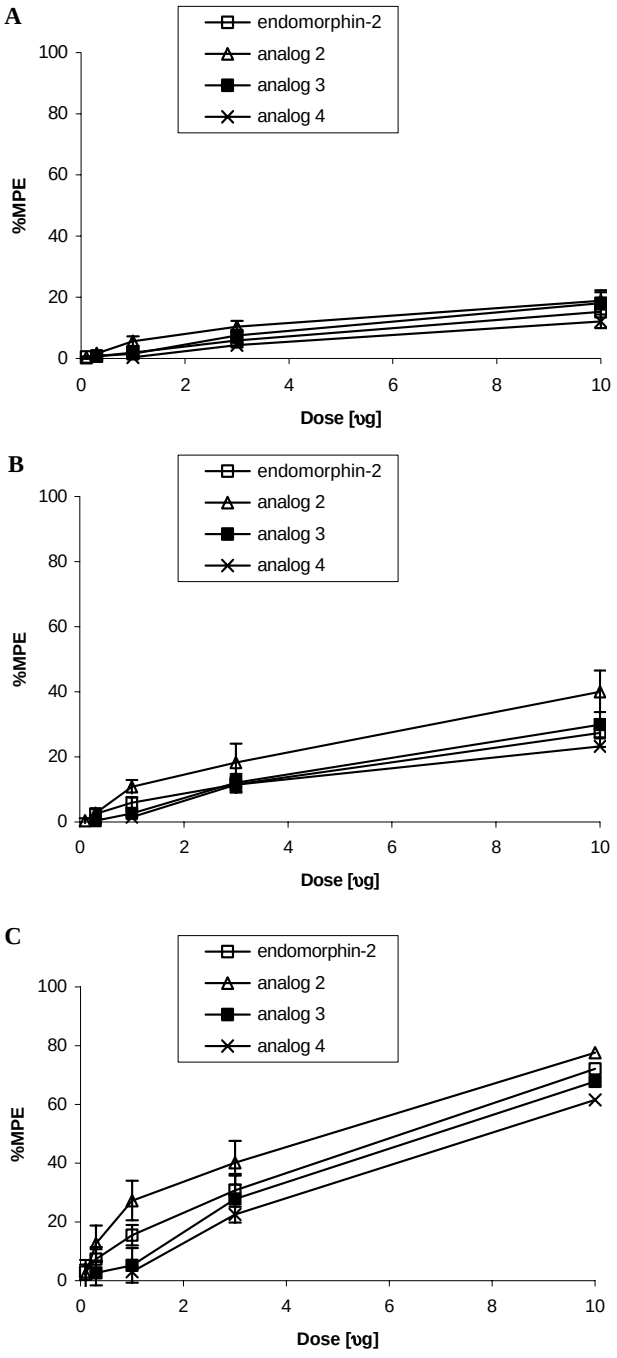

Figure 1. Dose–response curves for the hot-plate inhibition of paw licking (A), rearing (B), and jumping (C) induced by icv injection of endomorphin-2 and N-methylated analogs.

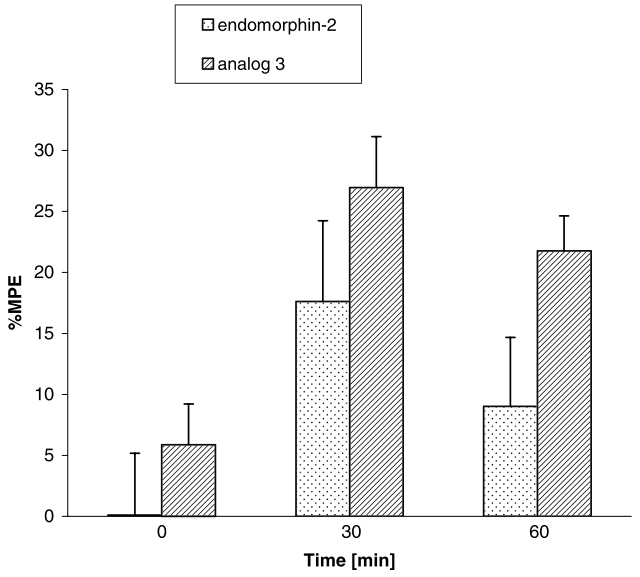

Figure 2. Time-course of the changes in the hot-plate inhibition of jumping induced by ip administration of endomorphin-2 and [Sar[2]]endomorphin-2.

agreement with those obtained in the hot-plate test of analgesia, which is consistent with the action of μ-opioid receptor agonists. Antinociceptive effect of [Sar^2]endomorphin-2 after icv injection resulted in a dose-dependent increase of the pain threshold. The effect observed after ip injection of this analog was slightly less pronounced.

In conclusion, the N-methylated analogs of endomorphin-2 described in this paper can be viewed as interesting models for the study of ligand–receptor interactions. μ-Opioid receptor affinity of [Sar^2]endomorphin-2 was comparable to that of the parent endomorphin-2. This analog was also equipotent with endomorphin-2 in triggering analgesia in mice after icv administration. When injected peripherally (ip) pain threshold was significantly increased, as compared with endomorphin-2 parent compound.

4. Experimental

4.1. Peptide synthesis

Peptides were synthesized by standard solid-phase procedures, as described before,[18] using techniques for Fmoc-protected amino acids on MBHA Rink peptide resin (100–200 mesh, 0.8 mM/g, Novabiochem). Piperidine (20%) in dimethylformamide was used for deprotection of Fmoc-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 trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5) for 3 h at room temperature. Crude peptides were purified by RP HPLC on a Vydac C_{18} column (1 × 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.

4.2. Opioid receptor binding assays

Receptor binding assay was performed, as described previously.[19] 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 ice-cold saline solution and the bound radioactivity was measured in the liquid scintillation counter L5 5000 TA (Beckman). Nonspecific binding was determined in the presence of naltrexone hydrochloride (10 mM). The data were analyzed by a nonlinear least-squares regression analysis computer program Prism Graph Pad.

4.3. Nociceptive tests

Male Swiss albino mice (CD1, Charles River), weighing 20–22 g, and male mice (Balb/c) 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 am to 7 pm 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.

Icv 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.[20] Intraperitoneal (ip) injections were carried out conventionally.[19]

The hot-plate test was performed according to the method of Eddy and Leimbach.[21] 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 icv 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 means ± SEM. 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 comparisons 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 cutoff time (t_2) was 240 s.

The median antinociceptive dose (ED_{50}) was calculated, according to the method of Litchfield and Wilcox.[22]

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