Synthesis and Characterization of Potent and Selective μ-Opioid Receptor Antagonists, [Dmt[1], D-2-Nal[4]]endomorphin-1 (Antanal-1) and [Dmt[1], D-2-Nal[4]]endomorphin-2 (Antanal-2)

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To synthesize potent antagonists of the μ-opioid receptor, we prepared a series of endomorphin-1 and endomorphin-2 analogues with 3-(1-naphthyl)-D-alanine (D-1-Nal) or 3-(2-naphthyl)-D-alanine (D-2-Nal) in position 4. Some of these analogues displayed weak antagonist properties. We tried to strengthen these properties by introducing the structurally modified tyrosine residue 2,6-dimethyltyrosine (Dmt) in place of Tyr[1]. Among the synthesized compounds, [Dmt[1], D-2-Nal[4]]endomorphin-1, designated antanal-1, and [Dmt[1], D-2-Nal[4]]endomorphin-2, designated antanal-2, turned out to be highly potent and selective μ-opioid receptor antagonists, as judged on the basis of two functional assays, the receptor binding assay and the hot plate test of analgesia. Interestingly, another analogue of this series, [Dmt[1], D-1-Nal[4]]endomorphin-1, turned out to be a moderately potent mixed μ-agonist/δ-antagonist.

Introduction

Opioid antagonists are indispensable tools in opioid research.[1] In fact, the major criterion for the classification of an agonist effect as being opioid receptor-mediated is the ability of naloxone (a universal opioid receptor nonpeptide antagonist) to antagonize this effect in a competitive manner. Receptor-type selective opioid antagonists of an alkaloid structure have been known and used in pharmacological studies for a long time. Compounds such as β-funaltrexamine (μ),[2] naltrindole (δ),[3] and nor-binaltorphimine (κ)[4] are selective antagonists with subnanomolar receptor binding affinity. The highly selective opioid peptide-derived antagonists are the δ antagonists TIPP[Ψ][5a] and Dmt-Tic[6,7] and the κ antagonist dynantin.[8]

The first peptide antagonists with high μ-receptor selectivity were developed by Hruby and co-workers in 1986 through modifications of somatostatin.[9] The cyclic, conformationally constrained octapeptides D-Phe-c(Cys-Tyr-D-Trp-X-Thr-Pen)-Thr−NH_2 where X represents a Lys, Orn, or Arg residue, whose

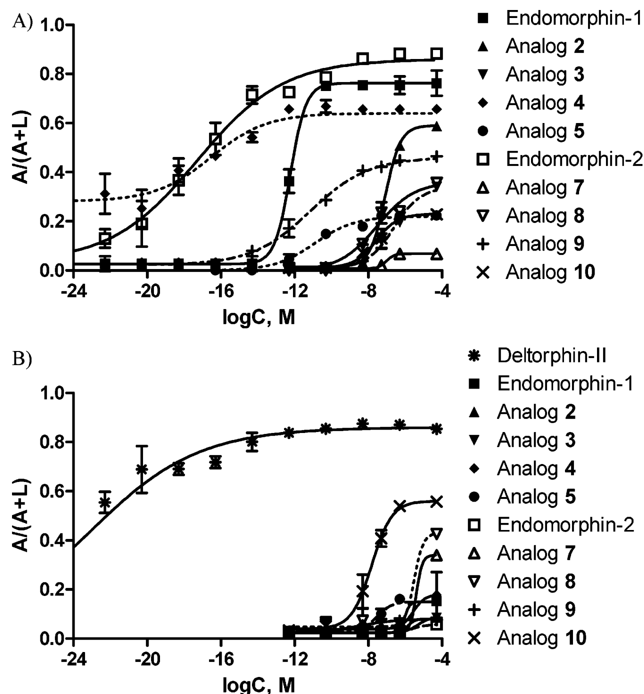

Figure 1. Concentration−response curves for the calcium increase induced by endomorphins and their analogues in the CHO-MOR-Aeq (A) and CHO-DOR-Aeq (B) cells. The data represent the mean ± SEM of three independent experiments carried out in duplicate.

structures are completely different from the structures of opioids, were for many years the only known peptide μ-antagonists. Recently, the first potent and selective opioid peptide-derived μ-antagonist, Dcp-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]−NH_2, has been reported.[10]

For several years, we have been trying to design selective antagonists of the μ-opioid receptor. In our earlier studies, we observed that the introduction of D-1-Nal or D-2-Nal into position 3 or 4 of endomorphin-2 led to analogues that showed substantial antagonist activity in the hot plate test in

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[a] Abbreviations: ANOVA, analysis of variance; CHO, Chinese hamster ovary; D-1-Nal, 3-(1-naphthyl)-D-alanine; D-2-Nal, 3-(2-naphthyl)-D-alanine; DAMGO, Tyr-D-Ala-Gly-N$^{\alpha}$MePhe-Gly-ol; Dcp, 3-(2,6-dimethyl-4-carbamoylphenyl)propanoic acid; DIPP−NH2[Y], Dmt-TicΨ[CH_2NH]Phe-Phe−NH_2; Dmt, 2′6′-dimethyltyrosine; DOR, δ-opioid receptor; DPDPE, Tyr-c[D-Pen-Gly-Phe-D-Pen]; DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr; Fmoc, 9-fluorenylmethoxycarbonyl; GPI, guinea pig ileum; i.c.v., intracerebroventricullar; MBHA, 4-methylbenzhydrylamine; MOR, μ-opioid receptor; MVD, mouse vas deference; RP HPLC, reversed-phase HPLC; TAPP, Tyr-D-Ala-Phe-Phe−NH_2; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; tBu, *t*-butyl; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIPP[Ψ], Tyr-Tic[CH_2-NH]-Phe-Phe; TIPP−NH_2, Tyr-Tic-Phe-Phe−NH_2; TIS, triisopropylsilane; β-FNA, β-funaltrexamine. The symbols and abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem J.* 1984, *219*, 345−373.

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Table 1. EC_{50} and pA_2 Values and Maximal Calcium Increases for the μ- and δ-Mediated Intracellular Calcium Response Induced by Endomorphin Analogues[a]

		CHO-MOR-Aeq			CHO-DOR-Aeq		
no.	sequence	EC_{50} ± SEM (nM)	pA_2[b]	max Ca^{2+}-increase (%)	EC_{50} ± SEM (nM)	pA_2[c]	max Ca^{2+}-increase (%)
1	Tyr-Pro-Trp-Phe−NH_2 (endomorphin−1)	0.001 ± 0.00001		86.05 ± 0.06	932 ± 11		15.31 ± 0.01
2	Tyr-Pro-Trp-D-1-Nal−NH_2	82.1 ± 1.4		58.67 ± 0.74	>1000		8.63 ± 0.10
3	Tyr-Pro-Trp-D-2-Nal−NH_2	248 ± 3		34.07 ± 0.23	>1000		8.46 ± 0.39
4	Dmt-Pro-Trp-D-1-Nal−NH_2	1.15 ± 0.02		64.05 ± 0.30		8.59	17.80 ± 9.29
5	Dmt-Pro-Trp-D-2-Nal−NH_2 (antanal-1)		7.61	22.36 ± 1.73	29.3 ± 0.5		15.24 ± 0.44
6	Tyr-Pro-Phe-Phe−NH_2 (endomorphin-2)	0.040 ± 0.001		85.89 ± 0.30	>1000		5.75 ± 0.11
7	Tyr-Pro-Phe-D-1-Nal−NH_2[d]		7.95	6.74 ± 0.19	>1000		33.96 ± 0.98
8	Tyr-Pro-Phe-D-2-Nal−NH_2[d]		6.42	35.75 ± 1.66	>1000		42.62 ± 0.60
9	Dmt-Pro-Phe-D-1-Nal−NH_2		9.00	46.45 ± 1.02	3.55 ± 0.07		7.63 ± 0.34
10	Dmt-Pro-Phe-D-2-Nal−NH_2 (antanal-2)		8.89	22.87 ± 0.02	15.5 ± 0.44		55.71 ± 0.58
	Tyr-D-Ala-Phe-Glu-Val-Val-Gly−NH_2 (deltorphin-II)				<0.01		86.59 ± 0.04
	Naloxone		9.66	0.10 ± 0.01			
	Naltrindole					7.95	

[a] Mean ± SEM of three independent experiments performed in duplicate. [b] Determined against endomorphin-2. [c] Determined against deltorphin-II. [d] Data from ref 22.

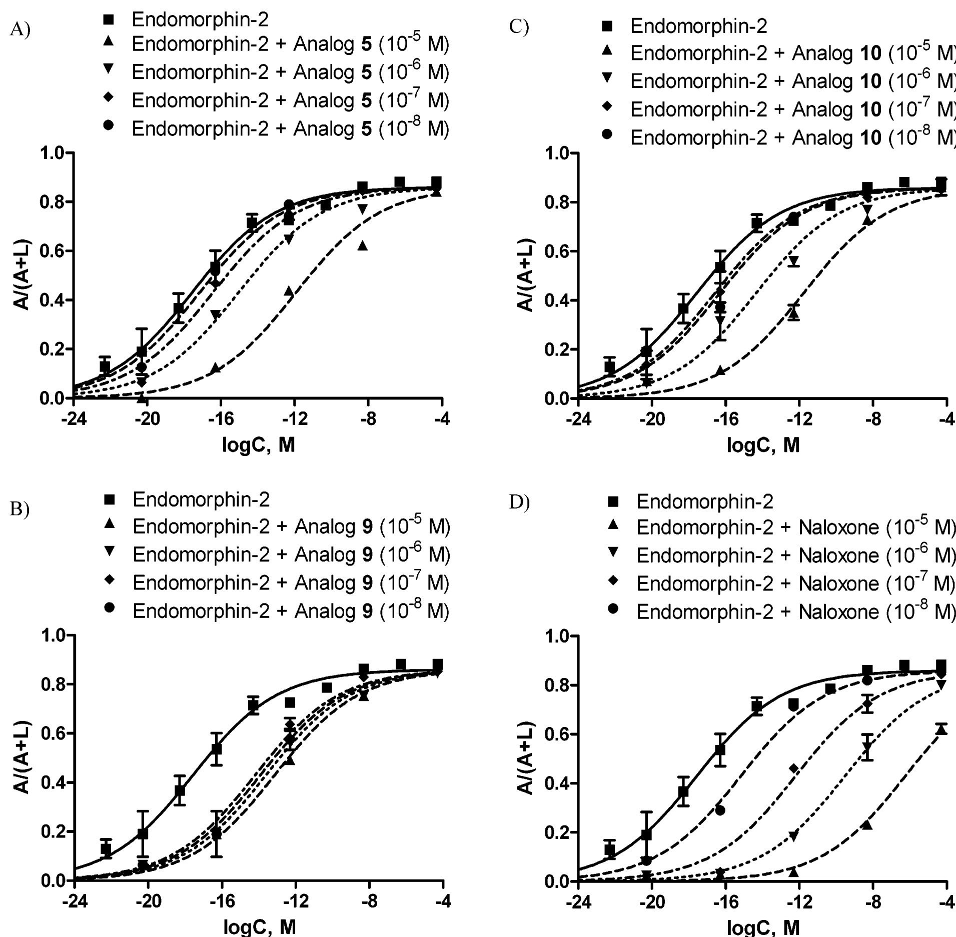

Figure 2. Concentration-dependent effect of [Dmt[1], D-2-Nal[4]]endomorphin-1 (**5**) (A), [Dmt[1], D-1-Nal[4]]endomorphin-2 (**9**) (B), [Dmt[1], D-2-Nal[4]]-endomorphin-2 (**10**) (C), and naloxone (D) on the concentration−response curves for the calcium rise induced by endomorphin-2 in the CHO-MOR-Aeq cells. The data represent the mean ± SEM of three independent experiments carried out in duplicate.

mice. The best antagonist analogue of that series was [D-1-Nal[4]]-endomorphin-2.[11] Because numerous studies[12−15] demonstrated that the substitution of Dmt for Tyr[1] in opioid peptides produces, in general, a pronounced increase in μ-opioid receptor affinity, we decided to investigate whether replacing the N-terminal Tyr residue with Dmt would strengthen the μ-antagonist properties of endomorphin-1 and endomorphin-2 analogues modified in position 4. Here, we report on the synthesis of four analogues of endomorphin-1 and four analogues of endomorphin-2 substituted either in position 4 (D-1-Nal or D-2-Nal) only or in

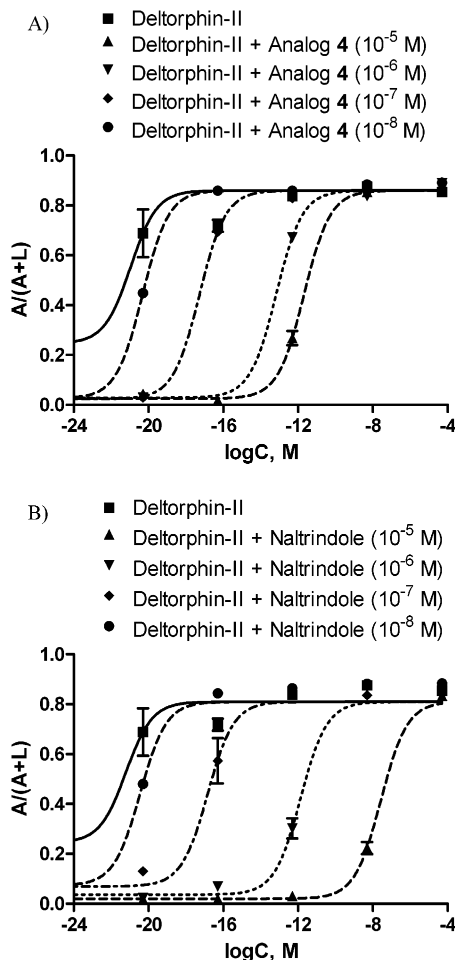

Figure 3. Concentration-dependent effect of [Dmt[1], D-1-Nal[4]]-endomorphin-1 (**4**) (A) and naltrindole (B) on the concentration−response curves for the calcium rise induced by deltorphin-II in the CHO-DOR-Aeq cells. The data represent the mean ± SEM of three independent experiments carried out in duplicate.

positions 1 (Dmt) and 4 (D-1-Nal or D-2-Nal). We also describe the opioid activities of these analogues as determined in two functional assays: peripheral smooth muscle assay on isolated guinea pig ileum (GPI) and mouse vas deferens (MVD) and the aequorin luminescence-based calcium assay. This functional assay based on aequorin-derived luminescence triggered by receptor-mediated changes in intracellular calcium levels, performed on recombinant cell lines, can be used instead of the traditional receptor binding assay to compare the affinity and selectivity of opioid ligands at μ- and δ-opioid receptors.[16] The analogues identified as μ-opioid receptor antagonists were also characterized in μ- and δ-receptor binding assay.

The antagonist effect of the new peptides was also studied in vivo on endomorphin-2- or DAMGO-induced analgesia in the hot plate test in mice.

Chemistry

Peptides were synthesized by the standard solid-phase method on MBHA Rink-Amide peptide resin using Fmoc-protected amino acids and TBTU as a coupling reagent. Peptides were cleaved from the resin by TFA/TIS treatment in the usual manner. Peptide purification was achieved by preparative RP HPLC.

Biology

Aequorin Luminescence-Based Calcium Assay. Analogues were tested in an assay based on the recombinant mammalian cell lines expressing the μ- or δ-opioid receptor together with the luminescent reporter protein, aequorin. Aequorin-derived luminescence, triggered by receptor-mediated changes in intracellular calcium levels, was used to examine the relative potency and efficacy of the opioid peptides. Apoaequorin is a 21 kDa photoprotein, isolated from the jellyfish *Aequorea victoria*, that forms a bioluminescent complex when linked to the chromophore cofactor coelenterazine.[17] The binding of Ca^{2+} to this complex results in an oxidation reaction of coelenterazine followed by the production of apoaequorin, coelenteramide, CO_2, and light with a λ_{max} of 469 nm, which can be detected by conventional luminometry.[18] In a cell line expressing recombinant apoaequorin, reconstitution of aequorin can be simply obtained by the addition of coelenterazine to the medium.[19]

In Vitro Assay on Isolated Tissue Preparations. Analogues were tested in a bioassay based on the inhibition of electrically evoked contractions of the guinea pig ileum (GPI) and the mouse vas deferens (MVD). The GPI assay is usually considered to be representative of μ-opioid receptor interactions, even though the ileum also contains κ-opioid receptors. In the MVD assay, opioid effects are primarily mediated by δ-opioid receptors, but μ- and κ-opioid receptors also exist in this tissue. Antagonist potencies in the GPI assay were determined against the μ-opioid agonist TAPP (Tyr-D-Ala-Phe-Phe−NH_2),[20] and the selective δ-opioid agonist DPDPE was used for δ-antagonist potency determinations in the MVD assay.

Opioid Receptor Binding Assay. Binding affinities of new analogues for the μ- and δ-opioid receptors were determined by displacing [^{3}H]DAMGO and [^{3}H]DSLET, respectively, from the rat brain membrane binding sites.

Analgesic Testing. 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 level appears to involve mainly δ-opioid receptor activation.[21] Drugs were admnistered to the mice by the i.c.v. route. Analgesic potencies were determined using the hot plate test. The latencies to paw licking, rearing, and jumping were measured.

Results

Aequorin Luminescence-Based Calcium Assay. Recombinant mammalian cell lines CHO-MOR-Aeq and CHO-DOR-Aeq expressing, respectively, the μ- or δ-opioid receptors and apoaequorin were used to study the agonist-induced bioluminescent responses caused by the release of intracellular Ca^{2+} ions. The concentration−response curves for endomorphins and their analogues in the μ- and δ-opioid-receptor expressing cells are depicted in Figure 1, parts A and B, respectively. The EC_{50} and pA_2 values and the maximal calcium rise for the μ- and δ-mediated intracellular calcium responses induced by endomorphins and their analogues are shown in Table 1. Endomorphin-1 and endomorphin-2 produced a potent agonist effect in the CHO-MOR-Aeq cells, with EC_{50} values of 0.001 ± 0.00001 and 0.040 ± 0.001 nM, respectively, and maximal Ca^{2+} increases of 86.05 ± 0.06 and 85.89 ± 0.30%, respectively. For comparison, deltorphin-II was used as a potent δ-agonist in the CHO-DOR-Aeq cell line (EC_{50} value <0.01 nM and a maximal Ca^{2+} stimulation of 86.59 ± 0.04%). Of the analogues tested, only [Dmt[1], D-1-Nal[4]]endomorphin-1 (**4**) was a weak partial agonist at the μ-opioid receptor (EC_{50} =

Table 2. GPI and MVD Assay of Endomorphin Analogues[a]

no.	sequence	GPI		MVD	
		IC_{50} (nM)	K_e (nM)[b]	IC_{50} (nM)	K_e (nM)[c]
1	Tyr-Pro-Trp-Phe−NH_2 (endomorphin-1)	17.2 ± 2.4		26.3 ± 3.9	
2	Tyr-Pro-Trp-D-1-Nal−NH_2	4180 ± 260		P.A. (e = 0.43)[d]	
3	Tyr-Pro-Trp-D-2-Nal−NH_2	3470 ± 260		IC_{30} = 4180 ± 1150[e]	
4	Dmt-Pro-Trp-D-1-Nal−NH_2	15.8 ± 1.1			62.4 ± 6.1
5	Dmt-Pro-Trp-D-2-Nal−NH_2 (antanal-1)		8.27 ± 0.84	P.A. (e = 0.37)[d]	
6	Tyr-Pro-Phe-Phe−NH_2 (endomorphin-2)	7.71 ± 1.47		15.3 ± 1.8	
7	Tyr-Pro-Phe-D-1-Nal−NH_2		1250 ± 40	>10 000	
8	Tyr-Pro-Phe-D-2-Nal−NH_2		1260 ± 50	inactive	
9	Dmt-Pro-Phe-D-1-Nal−NH_2		14.9 ± 0.8		227 ± 8
10	Dmt-Pro-Phe-D-2-Nal−NH_2 (antanal-2)		5.86 ± 0.78		250 ± 37

[a] Mean of three to five determinations ± SEM. [b] Determined against TAPP. [c] Determined against DPDPE. [d] Partial agonist. [e] Partial agonist (e = 0.60).

1.15 ± 0.02 nM and a maximal Ca^{2+} increase of 64.05 ± 0.30%). This analogue did not produce a significant effect in the δ-opioid receptor expressing cells. Analogues **2**, **3**, **5**, and **7**−**10** did not produce any significant calcium responses in either cell line.

In our earlier studies, we have shown that [D-1-Nal[4]]-endomorphin-2 (**7**) and [D-2-Nal[4]]endomorphin-2 (**8**) were μ-opioid receptor antagonists with pA_2 values of 7.95 and 6.42, respectively.[22] To examine the μ-antagonist properties of peptide analogues **2**, **3**, **5**, **9**, and **10** in the aequorin luminescence-based calcium assay, competition experiments against μ-selective agonist endomorphin-2 were performed. The pA_2 values were then calculated and compared with the value for naloxone, a well known universal opioid antagonist of the alkaloid structure. Analogues **5**, **9**, and **10** produced a concentration-dependent rightward shift of the concentration−response curve of endomorphin-2 (Figure 2). [Dmt[1], D-1-Nal[4]]-endomorphin-2 (**9**) and [Dmt[1], D-2-Nal[4]]endomorphin-2 (**10**) were the most potent peptide antagonists, with pA_2 values of 9.00 and 8.89, respectively. Both analogues were only slightly less potent than naloxone (pA_2 = 9.66). The rank order of the μ-opioid receptor antagonist potency was **9** > **10** > **7** > **5** > **8**.

In the δ-opioid receptor expressing cells, the action of deltorphin-II was antagonized by naltrindole, the δ-selective antagonist (pA_2 = 7.95), and analogue **4** (pA_2 = 8.59, Figure 3).

Functional Assay on Isolated Tissue Preparations (GPI/MVD). Of the endomorphin analogues tested in the functional GPI assay, [Dmt[1], D-1-Nal[4]]endomorphin-1 (**4**) was the most potent μ-opioid receptor agonist. This analogue was slightly more potent than the parent compound endomorphin-1 and only 2-fold less active than endomorphin-2 (Table 2). In the MVD assay, analogue **4** was a moderately potent δ-opioid antagonist. [Dmt[1], D-2-Nal[4]]endomorphin-1 (**5**), [Dmt[1], D-1-Nal[4]]endomorphin-2 (**9**), and [Dmt[1], D-2-Nal[4]]endomorphin-2 (**10**) were potent μ-opioid receptor antagonists and analogues **9** and **10** were also weak δ-receptor antagonists (K_e^{δ}/K_e^{μ} = 15.2 and 42.6, respectively). The most potent antagonist analogues **5** and **10** showed no agonist activity in the GPI assay at concentrations of up to 10 μM. Thus, they are pure μ-opioid receptor antagonists. Analogues **7** and **8** were weak μ-opioid receptor antagonists and were inactive at the δ-opioid receptor. The rank order of μ-antagonist potency in the GPI assay was **10** > **5** > **9** >> **7** > **8**. Analogues **2** and **3** were inactive in both functional assays.

Table 3. Opioid Receptor Binding Assay of the μ-Opioid Receptor Selective Antagonists[a]

no.	sequence	K_i^{μ} (nM)[b]	K_i^{δ} (nM)[c]	K_i^{δ}/K_i^{μ}
5	Dmt-Pro-Trp-D-2-Nal−NH_2 (antanal-1)	2.38 ± 0.33	17.4 ± 2.8	7.31
9	Dmt-Pro-Phe-D-1-Nal−NH_2	1.24 ± 0.12	27.1 ± 0.6	21.9
10	Dmt-Pro-Phe-D-2-Nal−NH_2 (antanal-2)	1.52 ± 0.38	7.74 ± 0.22	5.09

[a] Mean of three to four determinations. [b] Determined against [^{3}H]DAMGO. [c] Determined against [^{3}H]DSLET.

Opioid Receptor Binding Assay. In agreement with the GPI and MVD assay data, analogues **5**, **9**, and **10** showed nanomolar μ-opioid receptor binding affinities and weaker binding affinities for the δ-opioid receptors (Table 3). Among the three antagonists, analogue **9** showed the highest μ-opioid receptor selectivity (K_i^{δ}/K_i^{μ} = 21.9).

Antinociceptive Activity. In vivo studies on mice were performed to evaluate the functional assay results. Hot plate analgesia, which is known to be mediated mainly by the μ-opioid receptor, was studied. Latencies expressed as a percentage of the maximal possible effect (%MPE) to paw licking, rearing, and jumping are shown in Table 4. Peptides **2** and **3**, inactive in both functional assays, were not tested. Peptides **7** and **8** had been tested before and showed weak μ-antagonist activity.[11] Of the tested peptides, only analogue **4** displayed weak analgesic activity (Figure 4). The antagonist effect of analogues **5**, **9**, and **10** on endomorphin-2 (3 μg)-induced analgesia was studied and compared with that of β-funaltrexamine, a selective μ-opioid receptor non-peptide antagonist (Figure 5). Antinociception induced by endomorphin-2 was reversed by concomitant i.c.v. administration of any of these analogues, indicating that these analogues were μ-opioid antagonists. The antagonist effect of analogues **5**, **9**, and **10** on endomorphin-2- and DAMGO-induced analgesia in the hot plate test in mice was dose-dependent (Figures 6 and 7, respectively). The order of antagonist potency was **5** > **10** > **9**.

Discussion

In opioid peptides, two aromatic amino acids, Tyr[1] and either Phe[3]/Trp[3] or Phe[4], were found to be important structural elements that interact with opioid receptors.[23] Phe/Trp in position 3 or Phe in position 4 of endomorphins can be substituted with other aromatic amino acid residues, for example, naphtylalanines, without compromising receptor binding.[11] Recent structure−activity studies of opioid peptides have demonstrated that the

Table 4. Antinociceptive Effect of Selected Endomorphin Analogues in the Mouse Hot Plate Test after i.c.v. Injection (10 μg/animal)[a]

no.	sequence	latencies (%MPE) to		
		paw licking	rearing	jumping
1	Tyr-Pro-Trp-Phe−NH_2 (endomorphin-1)	5.99 ± 1.26	12.0 ± 2.6	40.8 ± 7.5
2	Tyr-Pro-Trp-D-1-Nal−NH_2			
3	Tyr-Pro-Trp-D-2-Nal−NH_2			
4	Dmt-Pro-Trp-D-1-Nal−NH_2	5.35 ± 1.02	10.6 ± 2.0	23.2 ± 4.4
5	Dmt-Pro-Trp-D-2-Nal−NH_2 (antanal-1)	0.17 ± 0.03	0.49 ± 0.07	0.58 ± 0.30
6	Tyr-Pro-Phe-Phe−NH_2 (endomorphin-2)	13.7 ± 2.3	24.8 ± 3.0	64.7 ± 8.0
7	Tyr-Pro-Phe-D-1-Nal−NH_2[b]	0.00 ± 0.02	0.31 ± 0.06	0.80 ± 0.22
8	Tyr-Pro-Phe-D-2-Nal−NH_2[b]	0.13 ± 0.03	0.09 ± 0.09	2.50 ± 0.32
9	Dmt-Pro-Phe-D-1-Nal−NH_2	0.13 ± 0.03	1.06 ± 0.60	2.73 ± 3.81
10	Dmt-Pro-Phe-D-2-Nal−NH_2 (antanal-2)	0.09 ± 0.03	0.18 ± 0.07	1.54 ± 0.32

[a] The data represent the mean ± SEM (n = 10). [b] Data from ref 7.

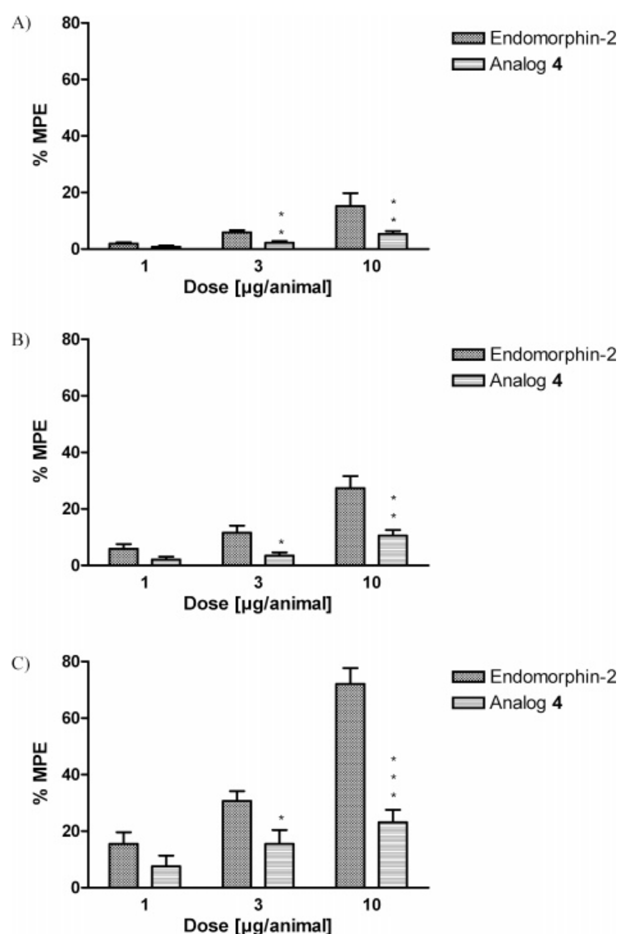

Figure 4. Dose−response effect of the hot plate inhibition of paw licking (A), rearing (B), and jumping (C) induced by i.c.v. injection of endomorphin-2 and [Dmt[1], D-1-Nal[4]]endomorphin-1 (**4**). The data represent the mean ± SEM of 10 mice per group. To calculate the statistical significance, we used one-way ANOVA and a post-hoc multiple-comparison Student Newman−Keuls test (*p < 0.05, **p < 0.01, and ***p < 0.001, significantly different from those of endomorphin-2 treated animals).

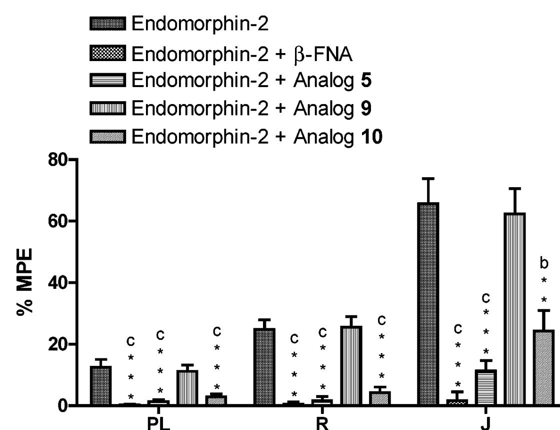

Figure 5. Comparison of the antagonist effect of β-FNA, [Dmt[1], D-2-Nal[4]]endomorphin-1 (**5**), [Dmt[1], D-1-Nal[4]]endomorphin-2 (**9**), and [Dmt[1], D-2-Nal[4]]endomorphin-2 (**10**) at a dose of 1 μg/animal on endomorphin-2 (3 μg/animal)-induced analgesia in the hot plate test in mice. PL, paw licking; R, rearing; J, jumping. The data represent the mean ± SEM of 10 mice per group. *p < 0.05, **p < 0.01, and ***p < 0.001 as compared to respective control by using two-way ANOVA followed by the Student Newman−Keuls' test.

introduction of Dmt in place of the Tyr[1] residue produces vastly improved opioid receptor affinity.[14,15,24−27] This finding encouraged us to further modify a series of endomorphin analogues with D-1-Nal and D-2-Nal substitutions in position 4 in order to enhance their antagonist potency. As it turned out, replacement of the Tyr[1] residue with Dmt in this series of endomorphin-1 and endomorphin-2 analogues led to compounds that showed 2 orders of magnitude higher μ-antagonist potency in the luminescence-based calcium assay and GPI assay and also reversed the analgesic effect induced by endomorphin-2 or DAMGO in the hot plate test. Out of four Dmt-containing analogues of endomorphins, three (**5**, **9**, and **10**) were found to be highly potent μ-antagonists in both in vitro assays and in the hot plate test, whereas analogue **4** was a μ-agonist/δ-antagonist. The highest antagonist potency was found for analogues with D-2-Nal[4] substitutions. [Dmt[1], D-2-Nal[4]]-endomorphin-1 was designated antanal-1 and [Dmt[1], D-2-Nal[4]]-endomorphin-2 was designated antanal-2. Analogues **5**, **9**, and **10** have μ-opioid receptor antagonist potencies and μ-opioid receptor selectivities similar to those of the recently reported μ-antagonist Dcp-c[D-Cys-Gly-Phe($p$$NO_2$)-D-Cys]−$NH_2$.[10] In comparison with the somatostatin-derived μ-antagonists, compounds **5**, **9**, and **10** and Dcp[D-Cys-Gly-Phe-($p$$NO_2$)-D-Cys]−$NH_2$ are nearly equipotent μ-antagonists but are less μ-selective.

An interesting exception in this Dmt-series of analogues was [Dmt[1], D-1-Nal[4]]endomorphin-1 (**4**), which turned out to be a μ-agonist/δ-antagonist. Compounds with such a profile are expected to be analgesics with a low propensity to produce tolerance and physical dependence and might be of benefit in the management of chronic pain.[28] The first known compound with mixed μ-agonist/δ-antagonist properties was the tetrapeptide amide TIPP−NH_2.[29] Substitution of Dmt for Tyr[1] in TIPP−NH_2 and reduction of the peptide bond between Tic[2] and Phe[3]

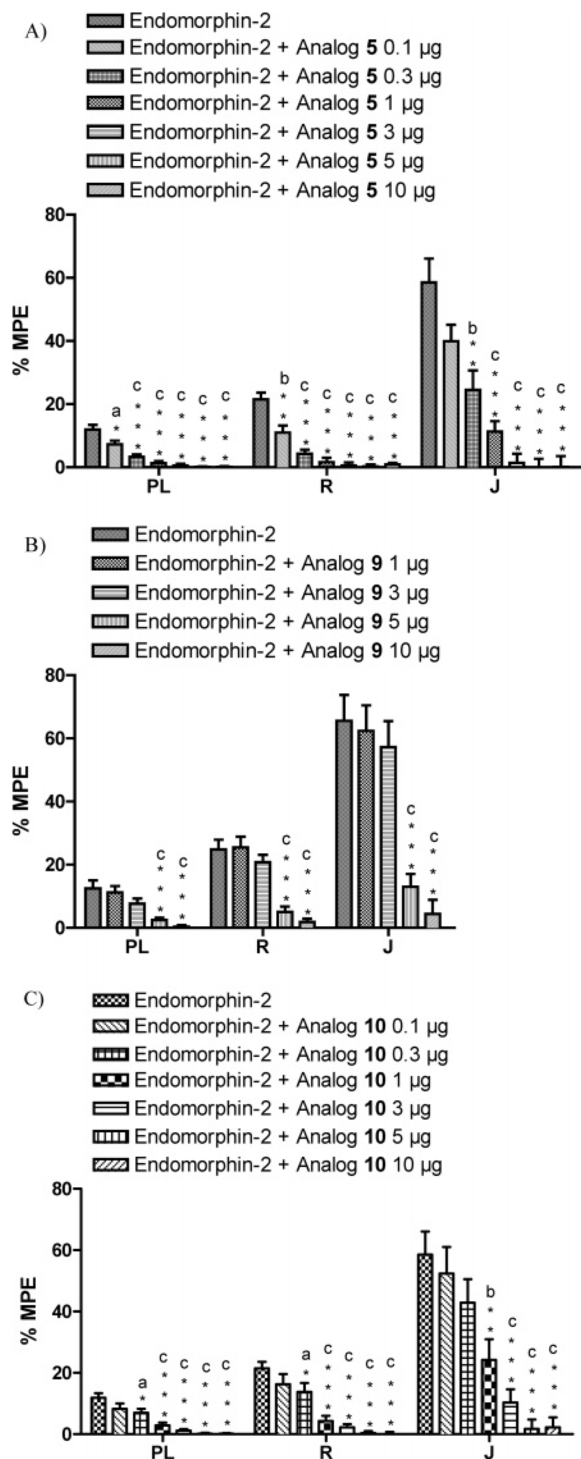

Figure 6. Dose-related antagonist effect of [Dmt[1], D-2-Nal[4]]endomorphin-1 (**5**) (A), [Dmt[1], D-1-Nal[4]]endomorphin-2 (**9**) (B), and [Dmt[1], D-2-Nal[4]]endomorphin-2 (**10**) (C) on endomorphin-2 (3 μg/animal)-induced analgesia in the hot plate test in mice. PL, paw licking; R, rearing; J, jumping. The data represent the mean ± SEM of 10 mice per group. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ as compared to respective control by using two-way ANOVA followed by the Student Newman–Keuls' test.

led to the compound DIPP–NH$_2$[Ψ],[25] which showed potent μ-agonist and δ-antagonist activities. DIPP–NH$_2$[Ψ] produced a potent antinociceptive effect in the rat tail-flick test, less tolerance than morphine, and no physical dependence after chronic administration at high doses.[25] Another peptide with a mixed μ-agonist/δ-antagonist profile was the cyclic

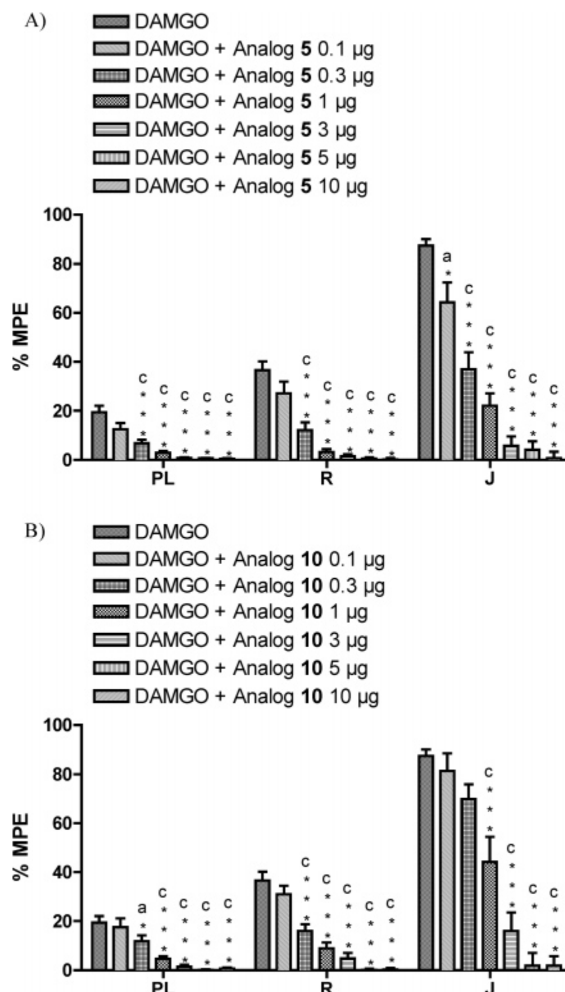

Figure 7. Dose-related antagonist effect of [Dmt[1], D-2-Nal[4]]endomorphin-1 (**5**) (A) and [Dmt[1], D-2-Nal[4]]endomorphin-2 (**10**) (B) on DAMGO (0.5 μg/animal)-induced analgesia in the hot plate test in mice. PL, paw licking; R, rearing; J, jumping. The data represent the mean ± SEM of 10 mice per group. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ as compared to respective control by using two-way ANOVA followed by the Student Newman–Keuls' test.

Table 5. Physicochemical Data of Endomorphin Analogues

analogue no.	TLC[a] A	TLC[a] B	HPLC t_r[b] (min)	FAB-MS formula	MW	$(M + H)^+$	purity (%)
1	0.72	0.74	13.20	$C_{34}H_{38}N_6O_5$	610	611	98
2	0.70	0.78	16.12	$C_{38}H_{40}N_6O_5$	660	661	98
3	0.71	0.80	16.12	$C_{38}H_{40}N_6O_5$	660	661	97
4	0.76	0.86	16.53	$C_{40}H_{44}N_6O_5$	688	689	98
5	0.64	0.83	16.09	$C_{40}H_{44}N_6O_5$	688	689	98
6	0.72	0.75	12.51	$C_{32}H_{37}N_5O_5$	571	572	98
7	0.71	0.75	15.91	$C_{36}H_{39}N_5O_5$	621	622	97
8	0.69	0.76	15.91	$C_{36}H_{39}N_5O_5$	621	622	97
9	0.77	0.81	16.67	$C_{38}H_{43}N_5O_5$	649	650	98
10	0.76	0.85	16.60	$C_{38}H_{43}N_5O_5$	649	650	98

[a] R_f values for TLC in solvent systems: (A) *n*BuOH/AcOH/water (4/1/2) and (B) *n*BuOH/pyridine/AcOH/water (60/20/6/24). [b] HPLC elution on a Vydac C_{18} column (0.46 × 25 cm^2, 5 μm) using the solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 20–80% solvent B over 25 min at flow rate of 1 mL/min.

β-casomorphin-5 analogue Tyr-c[D-Orn-2-Nal-D-Pro-Gly],[30] which contains the other isomer of naphthylalanine than our analogue **4**.

Conclusions

The structural modifications of endomorphin-1 and endomorphin-2 including the introduction of D-1-Nal or D-2-Nal into position 4 alone or simultaneously with the replacement of Dmt for Tyr[1] resulted in an interesting series of analogues. Three of the double-substituted analogues, [Dmt[1], D-2-Nal[4]]endomorphin-1, [Dmt[1], D-1-Nal[4]]endomorphin-2, and [Dmt[1], D-2-Nal[4]]-endomorphin-2 were very potent μ-antagonists in two functional assays (GPI and aequorin luminescence-based calcium assays) and in the hot plate test of analgesia in mice. D-2-Nal substitution in these analogues turned out to induce a higher antagonist potency than D-1-Nal substitution. The D-2-Nal-containing analogues [Dmt[1], D-2-Nal[4]]endomorphin-1 and [Dmt[1], D-2-Nal[4]]-endomorphin-2 were designated antanal-1 and antanal-2, respectively.

[Dmt[1], D-1-Nal[4]]endomorphin-1 was found to be a moderately potent mixed μ-agonist/δ-antagonist.

Experimental Section

Peptide Synthesis. Peptides were prepared by a manual solid-phase technique using Fmoc protection of the α-amino group and tBu protection of the Tyr side chain. TBTU was used as a coupling agent. The peptides were assembled on MBHA Rink-Amide peptide resin (100−200 mesh, 0.8 mM/g, Novabiochem, La Jolla, CA), according to a published protocol.[31] Piperidine (20%) in dimethylformamide was used for the deprotection of Fmoc groups. Simultaneous deprotection and cleavage from the resin were accomplished by treatment with TFA/TIS/water (95/2.5/2.5) for 3 h at room temperature. The resin was removed by filtration at a reduced pressure and washed twice with TFA. The combined filtrates were treated with a 10-fold volume of cold ether. The precipitated peptides were collected by filtration, washed with cold ether, dissolved in 1% TFA solution, and lyophilized. The crude peptides were purified by preparative RP HPLC on a Vydac C_{18} column (1.0 × 25 cm^2, 10 μm) equipped with a Vydac guard cartridge (W.R. & Co.-Conn., Columbia, MD). For purification, a linear gradient of 10−70% acetonitrile containing 0.1% TFA over 60 min at a flow rate of 2 mL/min was used. The purification was monitored at 214 nm.

The purity of the peptides was verified by ascending TLC performed on precoated plates (silica gel 60 F_{254}, 250 μm, Merck, Darmstadt, Germany) in the following solvent systems (all v/v): (A) *n*BuOH/AcOH/water (4/1/2) and (B) *n*BuOH/pyridine/AcOH/water (60/20/6/24). Three methods of visualization (ninhydrin, chlorine reagents, and UV absorption) detected a single spot after TLC in each solvent system. The analytical RP HPLC employed a Vydac C_{18} column (0.46 × 25 cm^2, 5 μm) and the solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B). A linear gradient of 20−80% solvent B for 25 min at a flow rate of 1 mL/min was used for the analysis. The absorbance was monitored at 214 nm. The final purity of the peptides was >97%. The calculated values for the protonated molecular ions were in agreement with those determined by FAB mass spectrometry. The physicochemical data of the analogues are summarized in Table 5.

Aequorin Assay. The luminescence-based calcium assay was performed as described before. Test compounds were dissolved in 50 μL of BSA medium and pipetted into the wells of the white 96-well plates. Light emission was recorded using a Microlumat Plus LB96V microplate luminometer (EG & G Berthold, Perkin-Elmer, Wellesley, MN) for 30 s immediately after the injection of a 50 μL cell suspension (i.e., 25 000 cells) into each well. Cells were then lysed by a second injection of 50 μL of 0.3% Triton X-100, followed by a 15 s monitoring period. Luminescence data (peak integration) were calculated using Winglow software (Perkin−Elmer, Wellesley, MN), which was linked to the Microsoft Excel program. Results are expressed as the fractional luminescence, that is, the ratio of the agonist-generated signal and the total luminescence (agonist + lysed cells), thereby correcting for potential well-to-well variation in the number of injected cells.

In Vitro Assays on Isolated Tissue Preparations. The GPI assay was performed as described before.[32] Male guinea pigs (300−450 g) were sacrificed by a blow to the skull. A 2- to 3-cm segment of ileum not less than 10 cm from the ileocecal junction was mounted in a 20 mL organ bath containing a modified Krebs' buffer of the following composition (mM): NaCl, 150; KCl, 4.3; $CaCl_2$, 1.25; $MgCl_2$, 1.0; $NaH_2PO_4xH_2O$, 1.7; $NaHCO_3$, 25.0; and glucose, 11.0. The buffer was saturated with 95% O_2/5% CO_2 and kept at 37 °C. The tissues were stimulated with 10 min^{-1} pulses of 4 ms duration. Contractions were recorded with a Hewlett-Packard (Berkeley Heights, CA) model FTA-1-1 force transducer connected to a Hewlett-Packard 7702B recorder. IC_{50} values (the concentration that suppresses the contraction by 50%) were determined by regression analysis of the concentration−response curve. The MVD assay was performed as described before.[32] Adult, male albino mice (Swiss Webster, 30−50 g) were sacrificed by cervical dislocation. Vasa deferentia were removed and suspended in a 5 mL organ bath containing a modified Krebs' buffer of the following composition (mM): NaCl, 118; KCl, 4.75; $CaCl_2$, 2.54; KH_2PO_4, 1.19; $NaHCO_3$, 25.0; glucose, 11.0; and L-tyrosine, 0.2. The buffer was saturated with 95% O_2/5% CO_2 and kept at 37 °C. The tissues were stimulated every 10 ms with 1 ms pulse trains of 0.15 Hz. The measurement of the reduction in the twitch height at various doses permitted the determination of the IC_{50} values.

Opioid Receptor Binding Studies. Opioid receptor binding experiments were performed as described in detail elsewhere.[32] Binding affinities for the μ- and δ-opioid receptors were determined by displacing, respectively, [^{3}H]DAMGO (Multiple Peptide Systems, San Diego, CA) and [^{3}H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites. Incubations were performed for 2 h at 0 °C with [^{3}H]DAMGO and [^{3}H]DSLET at respective concentrations of 0.72 and 0.78 nM. IC_{50} values were determined from log dose−displacement curves, and K_i values were determined from the obtained IC_{50} values by means of the Cheng and Prusoff equation[33] using values of 1.3 and 2.6 nM for the dissociation constants of [^{3}H]DAMGO and [^{3}H]DSLET, respectively.

In Vivo Antinociception Test. The procedures used in this study were in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and were 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, IFFA-CREDO/Charles River, Saint-Germain sur L'Arbresle, France) 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 a standard semisynthetic laboratory diet and tap water ad libitum under controlled environmental conditions (temperature, 22 ± 1°C; 7 a.m. to 7 p.m. light−dark cycle). The mice were tested only once and sacrificed immediately thereafter by decapitation. To determine the antinociceptive effects of the opioids, the hot plate test was used.

The i.c.v. injections 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 McCormick.[34] All of the drugs for i.c.v. administration were dissolved in 0.9% NaCl.

The hot plate test was performed according to the method of Eddy and Leimbach.[35] 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.

To evaluate the hot plate test responses, the control latencies (t_0) and test latencies (t_1) were determined after the 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.

Data Analysis. Statistical and curve-fitting analyses were performed using Prism 4.0 (GraphPad Software Inc., San Diego, CA). The data are expressed as means ± SEM. Differences between groups were assessed by one-way ANOVA followed by a post-hoc multiple comparison Student Newman−Keuls test. The antagonist effects in the combination experiments were analyzed using two-way 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 lower was considered to be statistically significant.

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Supporting Information Available: General methods, experimental details, and detailed statistical analysis for Figures 5−7. This material is available free of charge via the Internet at http://pubs.acs.org.

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