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[^{35}S]GTPγS binding stimulated by endomorphin-2 and morphiceptin analogs ☆

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Abstract

The ability of several μ-selective opioid peptides to activate G-proteins was measured in rat thalamus membrane preparations. The μ-selective ligands used in this study were three structurally related peptides, endomorphin-1, endomorphin-2 and morphiceptin, and their analogs modified in position 3 or 4 by introducing 3-(1-naphthyl)-D-alanine (D-1-Nal) or 3-(2-naphthyl)-D-alanine (D-2-Nal). The results obtained for these peptides in [^{35}S]GTPγS binding assay were compared with those obtained for a standard μ-opioid agonist DAMGO. [D-1-Nal^3]Morphiceptin was more potent in G-protein activation (EC_{50} value of 82.5 ± 4.5 nM) than DAMGO ($EC_{50} = 105 \pm 9$ nM). [D-2-Nal^3]Morphiceptin, as well as endomorphin-2 analogs substituted in position 4 by either D-1-Nal or D-2-Nal failed to stimulate [^{35}S]GTPγS binding and were shown to be potent antagonists against DAMGO. It seems that the topographical location of the aromatic ring of position 3 and 4 amino acid residues can result in a completely different mode of action, producing either agonists or antagonists.

Keywords: μ-Opioid receptor ligand; Agonist; Antagonist; Rat thalamus; G protein-coupled receptor; Functional assay

Endogenous opioid peptides mediate a number of physiological functions, including modulation of the sensation of pain, regulation of gastrointestinal motility, production and secretion of neuroendocrine hormones, and modulation of the immune system responses, through the activation of the specific membrane bound receptors.

Opioid binding sites belong to the superfamily of heterotrimeric guanine-nucleotide binding G protein-coupled receptors (GPCRs). In the resting state, guanosine diphosphate (GDP) is bound to the α-subunit of the G protein. Activation of the GPCR by an agonist leads to the dissociation of GDP from the protein, allowing guanosine triphosphate (GTP) to bind [1]. This, in turn, leads to the dissociation of the α- and βγ-subunits of the G-protein, which are then able to interact with the effector systems. The intrinsic GTPase activity of the G_α hydrolyses GTP to GDP and the α- and βγ-subunits of the G-protein reassociate. Opioid receptors are linked to the pertussis toxin-sensitive adenylyl-cyclase inhibitory G proteins G_i and G_o [2]. Their activation leads to the inhibition of adenylyl cyclase [3], stimulation of potassium channel conductance [4], and inhibition of calcium channels [5].

The family of endogenous opioid peptides has been divided into three major groups, based on the precursor molecules from which they are enzymatically cleaved [6]. The endorphins, enkephalins, and dynorphins are derived

☆ *Abbreviations:* Boc, butyloxycarbonyl; BSA, bovine serum albumin; GDP, guanosine diphosphate; GPCRs, G protein-coupled receptors; GTP, guanosine triphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; [^{35}S]GTPγS, guanylyl 5′-[γ-[^{35}S]thio]-triphosphate; MBHA, *p*-methylbenzhydrylamine; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

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from proopiomelanocortin, proenkephalin, and prodynorphin, respectively, and all share a common Tyr-Gly-Gly-Phe N-terminal sequence. Although selectivity of the enkephalins for the δ-receptor [7] and dynorphins for the κ-receptor [8] was demonstrated, no endogenous ligand was attributed to the μ-receptor until recently, although morphine and other opiates are known to act primarily at the μ-binding sites.

In 1997 novel endogenous opioid peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), were isolated from bovine frontal cortex [9]. They were reported to have extremely high affinity and selectivity for the μ-opioid receptor and therefore are considered its endogenous ligands. The structure of endomorphins is different from the structure of the typical opioids. They are tetrapeptides with Pro in the second position and they have amidated C-terminus. Endomorphins are structurally related to another μ-opioid peptide, morphiceptin (Tyr-Pro-Phe-Pro-NH_2) [10], isolated many years earlier from the milk protein digests.

The similarity of endomorphin-2 and morphiceptin structures, which differ only in the fourth position (Phe and Pro, respectively), prompted us to study the relative efficacies of these two peptides and their position 3 and 4 modified analogs in stimulating the guanylyl 5′-[γ-[^{35}S]thio]-triphosphate ([^{35}S]GTPγS) binding and to compare the results with the data obtained in a classical binding assay.

Materials and methods

Peptide synthesis. Peptides were synthesized by a standard solid-phase procedure as described before [11], using techniques for butyloxycarbonyl (Boc)-protected amino acids on *p*-methylbenzhydrylamine (MBHA) resin (100–200 mesh, 0.8 mM/g, Novabiochem, La Jolla, USA). 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 hydrofluoric acid and 10% anisole scavenger at 0 °C for 1 h. 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.

Animals. The procedures used in this study were approved by the Local Ethical Committee. Male Wistar rats (Charles River, Saint-Germain sur l'Abresle, France), weighing 200–250 g, were used throughout the study. The animals were housed 5 per Makrolon box (L:40, W:25, H:18 cm), with free access to a 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).

Opioid receptor binding assays. Receptor binding assay was performed as described previously [12]. 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). 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 L5 5000 TA (Beckman, USA). Non-specific binding was determined in the presence of naltrexone hydrochloride (10 mM). Four independent experiments for each assay were carried out in duplicate.

[^{35}S]GTPγS binding assay. Crude membrane preparations were isolated according to the modified method described elsewhere [13]. Rats were sacrificed by decapitation. Thalamus and spinal cord were removed and homogenized in 20 vol. of 0.32 M sucrose. The homogenates were centrifuged (3000*g* for 15 min), the supernatants from two centrifugations were combined and centrifuged (13,500*g* for 30 min). The resulting pellet was then suspended in Tris buffer (50 mM Tris/HCl, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.4), sonicated, and centrifuged (10,000*g* for 10 min). The final protein concentration, determined by the method of Lowry [14], was about 1 mg/ml.

[^{35}S]GTPγS binding assays were performed according to the modified method described elsewhere [13]. Membranes (100 μg) were incubated at 25 °C for 2 h in the assay buffer with the appropriate concentration of tested peptide and/or with GDP (Sigma–Aldrich Co.) in the presence of 0.05 nM [^{35}S]GTPγS (1250 Ci/mmol; NEN Brussels, Belgium) in a total volume of 1 ml. Basal binding was assessed in the absence of peptide analog and presence of GDP, and the non-specific binding was assessed in the presence of 10 μM guanosine-5′-*O*-(3-thio)triphosphate (GTPγS; Sigma–Aldrich).

The entire mixture was incubated at 25 °C for 2 h and filtered through Whatman GF/B glass fiber filters, which had been pre-soaked for 2 h in Tris buffer, and washed three times with 4 ml of ice-cold Tris buffer, using a Millipore Sampling Manifold (Billerica, MA, USA). Bound radioactivity was determined in Tri-Carb 2100 TR liquid scintillation counter (Packard) after overnight extraction of the filters in 4 ml of Ultima Gold scintillation fluid (Perkin-Elmer). Four independent experiments for each assay were carried out in duplicate.

Statistical analysis. The data are expressed as means ± SEM. All statistical and curve-fitting analyses were performed using the computer software Prism 4.0 (GraphPad Software Inc., USA). The percent stimulation of [^{35}S]GTPγS binding was calculated according to the following formula: $(S - B)/B \times 100\%$, where S is the stimulated level and B is the basal level of [^{35}S]GTPγS binding. Individual dose–response curves were obtained by a non-linear regression analysis.

The K_e values for naloxone and antagonist peptides, as determined from rightward shifts of the agonist concentration–response curve, were calculated according to the Schild formula using a single concentration of competitive antagonist [15]: $K_e = [\text{Ant}]/(\text{DR}-1)$, where [Ant] is the concentration of the antagonist and DR is the ratio of the EC_{50} values of an agonist in the presence and absence of antagonist.

Statistical comparison between experimental conditions was assessed by analysis of variance (ANOVA) followed by Student–Newman–Keuls test. A probability level of 0.05 or smaller was used to indicate statistical significance.

Results

The competitive radioligand binding experiments and μ-opioid receptor activation of the G-proteins in the functional [^{35}S]GTPγS assays were used to compare the binding characteristics of several μ-opioid selective peptides and their analogs. The μ-selective ligands used in this study were Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO), structurally related peptides: endomorphin-1, endomorphin-2, and morphiceptin, and the analogs of endomorphin-2 and morphiceptin modified in position 3 or 4 by introducing 3-(1-naphthyl)-D-alanine (D-1-Nal) or 3-(2-naphthyl)-D-alanine (D-2-Nal).

μ-Receptor binding affinities, as measured by IC_{50} values against [^{3}H]naloxone, are provided in Table 1. The introduction of D-1-Nal residue in position 3 of endomorphin-2 and morphiceptin produced opposite results: an

Table 1
μ-Opioid receptor binding affinities of μ-selective opioid peptides and their analogs

No.	Peptide	IC_{50}[a] (nM)	Relative potency
1	DAMGO	1.22 ± 0.12	1
2	Endomorphin-1	0.97 ± 0.22	1.26
3	Endomorphin-2	3.90 ± 0.20	0.31
4	Morphiceptin	79.4 ± 3.4	0.02
5	[D-1-Nal^3]Endomorphin-2	89.1 ± 3.8	0.01
6	[D-1-Nal^4]Endomorphin-2	14.0 ± 1.25	0.09
7	[D-2-Nal^4]Endomorphin-2	19.5 ± 2.10	0.06
8	[D-1-Nal^3]Morphiceptin	1.90 ± 0.20	0.64
9	[D-2-Nal^3]Morphiceptin	158 ± 11	<0.01
10	[Dmt^1, D-1-Nal^3]Morphiceptin	0.33 ± 0.02	3.70

[a] Displacement of [^{3}H]naloxone. The data represent means ± SEM of four independent experiments carried out in duplicate.

about 20-fold increase and about 40-fold decrease in the IC_{50} values, respectively (peptides **5** and **8**). The highest affinity at the μ-receptor (IC_{50} value of 0.33 ± 0.02 nM) was found for analog **10**, which was [D-1-Nal^3]morphiceptin additionally modified in position 1 by introducing 2′,6′-dimethyltyrosine (Dmt). The D-2-Nal modification in position 3 of morphiceptin gave peptide with much lower affinity at the μ-opioid receptor. Endomorphin-2 analogs, modified in position 4, [D-1-Nal^4]endomorphin-2 **6**, and [D-2-Nal^4]endomorphin-2 **7**, were an order of magnitude less potent than the parent compound.

For [^{35}S]GTPγS assays, two brain structures, rat thalamus and spinal cord, both rich in μ-opioid binding sites, were selected. First, the potency of DAMGO in stimulating [^{35}S]GTPγS binding in the membrane preparations obtained from these two structures was compared in the presence of 10 μM GDP. In both cases, DAMGO stimulated [^{35}S]GTPγS binding in a dose-dependent and saturable manner (Fig. 1), but the stimulation of [^{35}S]GTPγS binding in the thalamic membranes was significantly higher than in the spinal cord membranes (EC_{50} = 70.8 ± 5.4 and 501 ± 38 nM, respectively). Thalamic membranes were selected for further experiments.

Previous studies demonstrated that a large excess of GDP is necessary to produce a significant agonist-stimulated [^{35}S]GTPγS binding [16,17]. We have determined the appropriate concentration of GDP for detecting agonist-stimulated [^{35}S]GTPγS binding by incubating thalamic membrane preparations with various concentrations of GDP (Fig. 2). GDP (0.01–100 μM) inhibited [^{35}S]GTPγS binding in a concentration-dependent manner, with <7% of total [^{35}S]GTPγS bound with 100 μM GDP (Fig. 2A). Addition of the μ-opioid agonist DAMGO (10 μM) resulted in an increase in [^{35}S]GTPγS binding. DAMGO produced a weak effect in the presence of 0.01 μM GDP and the relative stimulation increased with the increasing concentration of GDP. A significant effect was observed at GDP concentration over 1 μM (Fig. 2B). The concentration of GDP selected for further experiments was 30 μM, which was required for the optimal stimulation of the [^{35}S]GTPγS binding by an agonist.

The ability of the μ-opioid receptor agonists: endomorphin-1 **2**, endomorphin-2 **3**, and morphiceptin **4** to

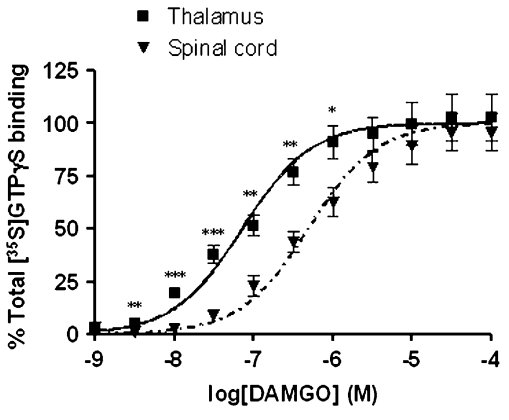

Fig. 1. Concentration-effect curve of DAMGO-stimulated G-protein activity in rat thalamus and spinal cord membrane preparations. The data represent means ± SEM of four independent experiments carried out in duplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as compared to respective control (% total [^{35}S]GTPγS binding in the spinal cord membrane preparations) by using one-way ANOVA followed by the Student–Newman–Keuls test.

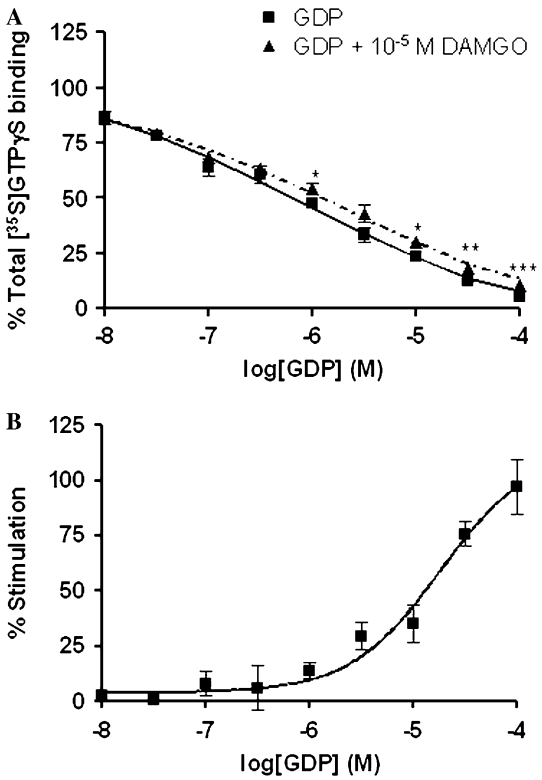

Fig. 2. Effect of GDP on basal and μ-opioid stimulated [^{35}S]GTPγS binding in rat thalamic membranes. (A) GDP concentration-effect curve of DAMGO-stimulated G-protein activity in rat thalamic membranes. (B) DAMGO-stimulated [^{35}S]GTPγS binding expressed as percentage stimulation at each concentration of GDP. The data represent means ± SEM of four independent experiments carried out in duplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as compared to respective control (% total [^{35}S]GTPγS binding without DAMGO) by using one-way ANOVA followed by the Student–Newman–Keuls test.

stimulate [^{35}S]GTPγS binding was measured and compared to the stimulation produced by DAMGO (Fig. 3). All these ligands stimulated [^{35}S]GTPγS binding in a dose-dependent and saturable manner. Next, the concentration–response curves for the analogs of endomorphin-2 and morphiceptin were obtained and compared with that for DAMGO. [D-1-Nal^3]Endomorphin-2 **5**, [D-1-Nal^3]morphiceptin **8**, and [Dmt^1, D-1-Nal^3]morphiceptin **10** were μ-receptor agonists (Fig. 4). [D-1-Nal^4]endomorphin-2 **6**, [D-2-Nal^4]endomorphin-2 **7**, and [D-2-Nal^3]morphiceptin **9** failed to stimulate [^{35}S]GTPγS binding in the membrane preparations from rat thalamus.

The EC_{50} values for DAMGO, endomorphin-1 **2**, endomorphin-2 **3**, morphiceptin **4**, and the analogs **5**, **8**, and **10** are presented in Table 2. [D-1-Nal^3]morphiceptin **8** was shown to be the most potent analog with the EC_{50} value of 82.5 ± 4.5 nM. The peptide agonists used in the study produced 86.76–133.68% of [^{35}S]GTPγS binding stimulated by DAMGO. Endomorphin-1 **2**, endomorphin-2 **3**, and morphiceptin **4** were all μ-receptor partial agonists compared to DAMGO. The analogs **5**, **8**, and **10** were shown to be full agonists (% maximal effect of DAMGO = 136.64 ± 8.40, 101.20 ± 17.08, and 133.68 ± 1.45, respectively).

To prove that DAMGO and the most potent analogs of endomorphin-2 and morphiceptin, [D-1-Nal^3]endomorphin-2 **5** and [D-1-Nal^3]morphiceptin **8**, respectively, stimulate [^{35}S]GTPγS binding through the μ-opioid receptor, concentration–response curves were obtained for these peptides in the presence of a competitive μ-receptor antagonist, naloxone (Fig. 5A–C). Naloxone produced rightward shifts of the concentration-response curves of the tested agonist peptides, which confirms the involvement of the μ-opioid receptor in the stimulation of the

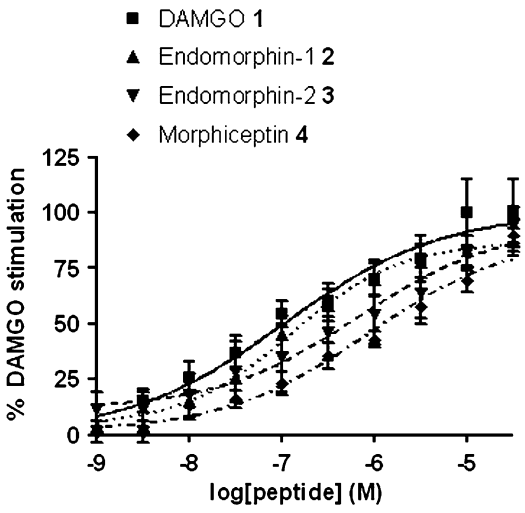

Fig. 3. Concentration-effect relationship of [^{35}S]GTPγS binding in rat thalamic membranes stimulated by μ-opioid receptor selective ligands: DAMGO **1**, endomorphin-1 **2**, endomorphin-2 **3**, and morphiceptin **4**. The data, expressed as stimulation relative to the maximal effect produced by DAMGO, represent means ± SEM of four independent experiments carried out in duplicate.

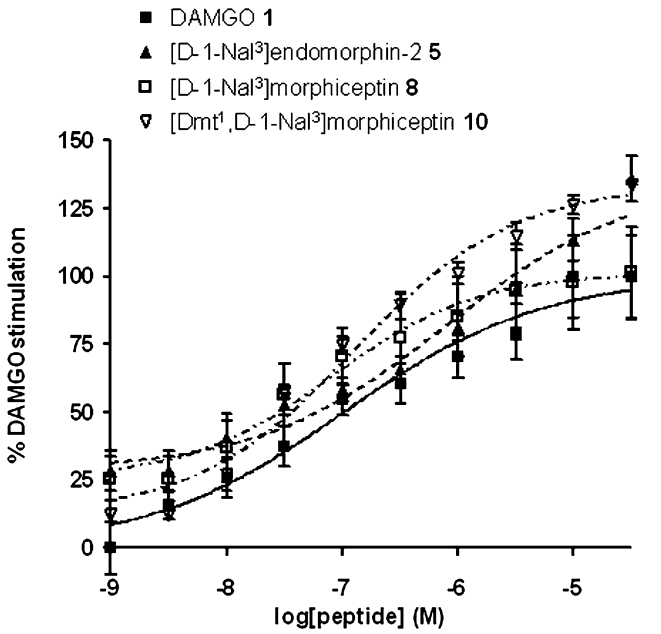

Fig. 4. Concentration-effect relationship of [^{35}S]GTPγS binding in rat thalamic membranes stimulated by μ-opioid receptor selective ligand DAMGO and the analogs of endomorphin-2 and morphiceptin: [D-1-Nal^3]endomorphin-2 **5**, [D-1-Nal^3]morphiceptin **8**, and [Dmt^1, D-1-Nal^3]morphiceptin **10**. The data, expressed as stimulation relative to the maximal effect produced by DAMGO, represent means ± SEM of four independent experiments carried out in duplicate.

[^{35}S]GTPγS binding by these ligands. The strongest antagonist effect of naloxone was observed for [D-1-Nal^3]endomorphin-2 **5** (K_e = 1.72 nM).

To determine whether the lack of ability of compounds **6**, **7**, and **9** to stimulate [^{35}S]GTPγS binding reflected their lack of potency, or antagonist activity at the μ-receptor, competition experiments against DAMGO were performed. The K_e values based on the rightward shift were then calculated and compared with naloxone. All tested analogs shifted the dose–response curve of DAMGO to the right, which indicates that they had antagonist properties at the μ-receptor (Fig. 6A–D). The most potent μ-receptor antagonist was [D-1-Nal^4]endomorphin-2 **6** with a K_e value of 2.80 ± 0.25 nM (Table 2). The rank order of antagonist activity was **6** > naloxone > **7** > **9**.

Discussion

Since their discovery in 1997, endomorphins are probably the most extensively studied opioid peptides. Many analogs of endomorphins have been synthesized in an attempt to develop new peptide analgesics, as well as to determine their pharmacological properties. The presence of an aromatic amino acid in positions 3 and 4 is crucial for an effective binding to the μ-opioid receptor [18]. A close structural similarity of endomorphin-2 and another atypical opioid peptide, morphiceptin, which both have a Phe residue in the third position, encouraged us to study and compare binding properties and antinociceptive

Table 2
Agonist activity of μ-selective opioid peptides and their analog in [^{35}S]GTPγS binding assay in rat thalamus membranes

No.	Peptide	EC_{50} (nM)	K_e[a] (nM)	Maximal effect (% max DAMGO)
1	DAMGO	105 ± 9	—	100.00 ± 15.25
2	Endomorphin-1	126 ± 4	—	86.76 ± 6.09
3	Endomorphin-2	657 ± 19	—	94.23 ± 11.98
4	Morphiceptin	857 ± 81	—	89.85 ± 7.46
5	[D-1-Nal^3]Endomorphin-2	803 ± 60	—	136.64 ± 8.40
6	[D-1-Nal^4]Endomorphin-2	—	2.80 ± 0.25	4.90 ± 3.25
7	[D-2-Nal^4]Endomorphin-2	—	5.30 ± 0.51	6.24 ± 1.01
8	[D-1-Nal^3]Morphiceptin	82.5 ± 4.5	—	101.20 ± 17.08
9	[D-2-Nal^3]Morphiceptin	—	10.6 ± 1.1	2.89 ± 1.55
10	[Dmt^1, D-1-Nal^3]Morphiceptin	127 ± 17	—	133.68 ± 1.45
	Naloxone	—	3.69 ± 0.31	21.65 ± 5.15

[a] Determined against DAMGO. The data represent means ± SEM of four independent experiments carried out in duplicate.

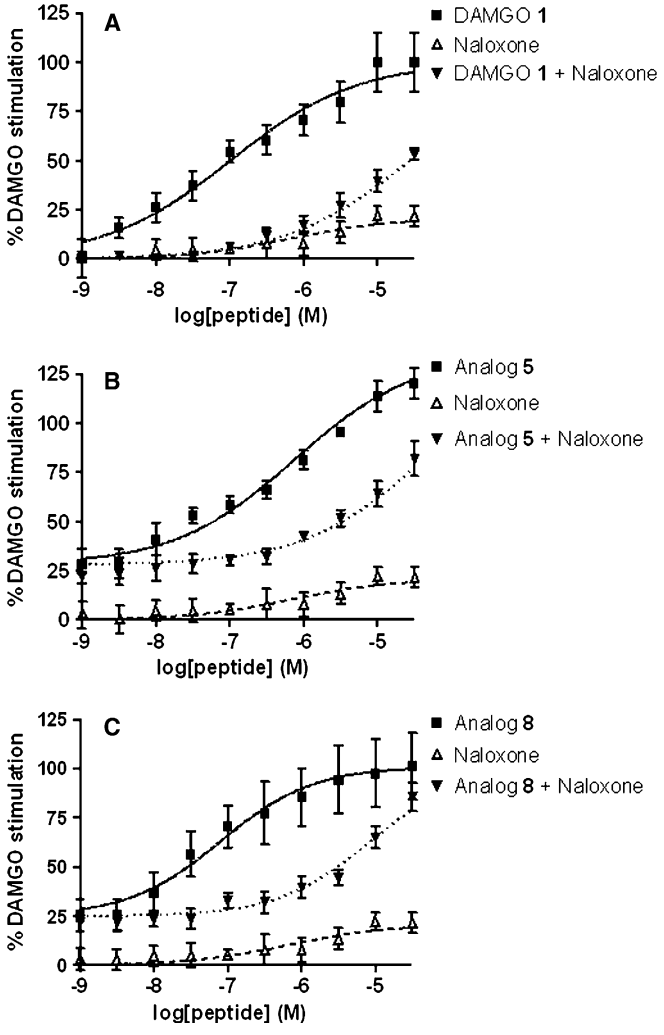

Fig. 5. Concentration-effect relationship of [^{35}S]GTPγS binding in rat thalamic membranes stimulated by DAMGO **1** (A), [D-1-Nal^3]endomorphin-2 **5** (B), and [D-1-Nal^3]morphiceptin **8** (C) in the absence and presence of 1 μM μ-receptor antagonist naloxone. The data, expressed as stimulation relative to the maximal effect produced by DAMGO, represent means ± SEM of four independent experiments carried out in duplicate.

activity of these two peptides and their analogs. In our previous papers [19,20] we investigated the in vivo analgesia of centrally administered endomorphin-2, morphiceptin, and their analogs, modified by introducing unnatural aromatic amino acids in positions 3 or 4. The most interesting results were obtained for D-1-Nal substitutions. Here we studied the properties of endmorphin-1, endomorphin-2, morphiceptin, and their analogs in the functional [^{35}S]GTPγS binding assay. This assay measures the level of G-protein activation following agonist or antagonist occupation of a G-protein coupled receptor. The great advantage of the assay is that it measures functional consequence of receptor occupancy by a ligand and it could be used to provide pharmacological parameters of potency, efficacy, and antagonist affinity. The correlation between intrinsic activity of a ligand in this assay and its efficacy in vivo makes it an appropriate model for measuring the relative potencies of opioid peptides.

In our study, we have examined the [^{35}S]GTPγS binding stimulated by structurally related peptides, endomorphin-1, endomorphin-2, and morphiceptin, and their analogs in the thalamic membrane preparations and compared the results with those obtained for DAMGO. DAMGO is a pentapeptide, whose sequence was derived from enkephalins [21] almost 25 years ago, and which for the last two decades was the most widely used μ-specific ligand with full agonist properties. In the present study, only endomorphin-2 was shown to be a full μ-opioid receptor agonist in the [^{35}S]GTPγS binding assay, while two other well-known μ-opioid peptide ligands, endomorphin-1 and morphiceptin, exhibited partial agonism. Sim et al. [22] already assayed the effect of endomorphin-1 on [^{35}S]GTPγS binding and demonstrated that it was also a partial agonist. The results of both studies show that the aromatic ring of the residue in position 3 of endomorphin-1 and endomorphin-2 (Trp and Phe, respectively), as well as the residue in position 4 (Phe in endomorphins and Pro in morphiceptin) plays an important role in the interaction of these peptides with the μ-opioid receptors.

Endomorphin-2 and morphiceptin analogs, containing D-1-Nal^3 and D-2-Nal^3 modifications exhibited completely different modes of action. [D-1-Nal^3]endomorphin-2 and [D-1-Nal^3]morphiceptin were both agonists, which is in good agreement with the results of the antinociceptive tests performed earlier for these analogs [19]. Morphiceptin

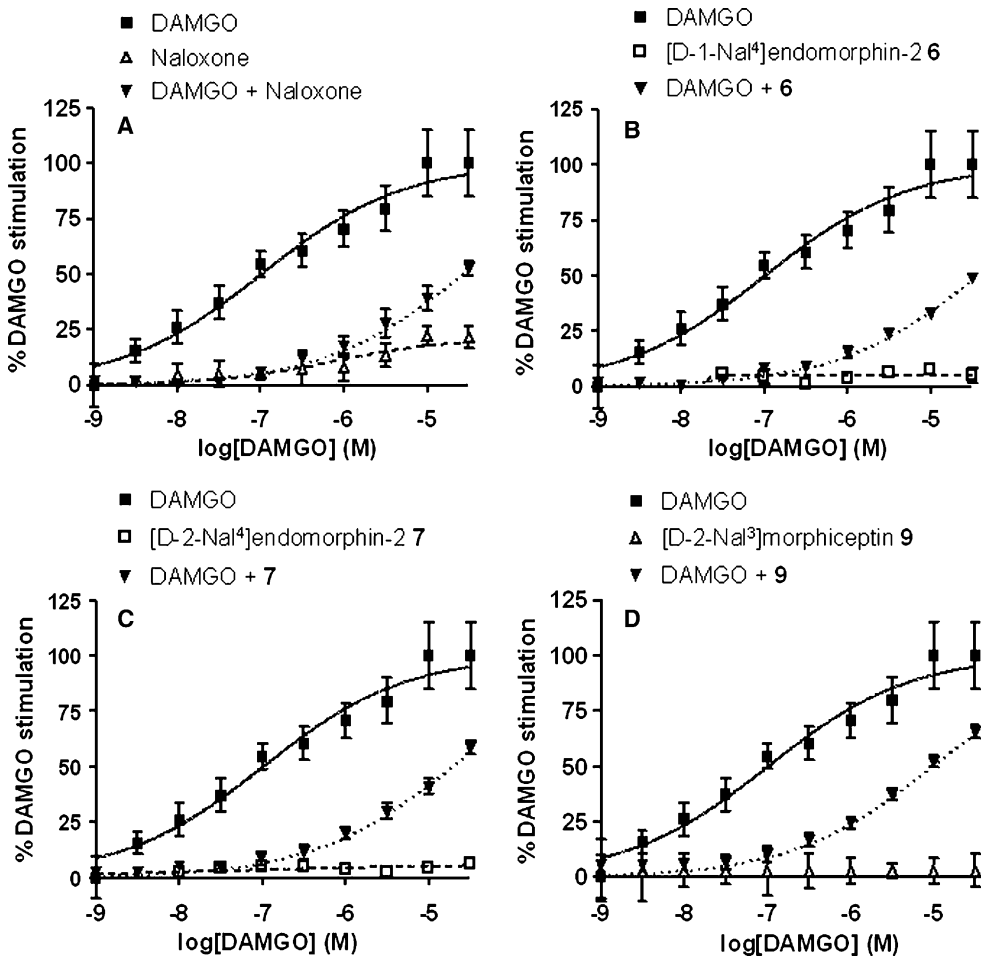

Fig. 6. Concentration-effect relationship of [^{35}S]GTPγS binding in rat thalamic membranes stimulated by DAMGO **1** in the absence and presence of 1 μM naloxone (A), [D-1-Nal^4]endomorphin-2 **6** (B), [D-2-Nal^4]endomorphin-2 **7** (C), or [D-2-Nal^3]morphiceptin **9** (D). The data, expressed as stimulation relative to the maximal effect produced by DAMGO, represent means ± SEM of four independent experiments carried out in duplicate.

analog containing [D-2-Nal] residue in position 3 exhibited antagonist activity.

Recent structure–activity studies have shown that the introduction of the unnatural amino acid 2′,6′-dimethyltyrosine (Dmt) in place of the N-terminal Tyr residue is a promising approach to enhance receptor affinity and functional potency [23]. [Dmt^1]endomorphin-2 was shown to be a potent μ-opioid receptor agonist [24]. In the present study, [Dmt^1, D-1-Nal^3]morphiceptin was found to be a potent μ-opioid receptor agonist, both in traditional binding studies and in the [^{35}S]GTPγS binding.

The introduction of D-1-Nal and D-2-Nal residue in position 4 of endomorphin-2 gave μ-receptor antagonists. The mode of action of these analogs in the [^{35}S]GTPγS binding assay is in good agreement with that observed in the antinociceptive test in vivo in mice after central and peripheral co-administration with a μ-receptor agonist, endomorphin-2 [20].

In conclusion, the topographical location of the aromatic ring of the position 3 and 4 amino acid residue seems to be critical not only for the binding affinity, but also for the stimulation of the [^{35}S]GTPγS binding and the activation of the downstream effector systems.

Acknowledgments

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