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

Jakub Fichna [a], Jean-Claude do-Rego [b], Piotr Kosson [c], Peter W. Schiller [d], Jean Costentin [b], Anna Janecka [a,*]

[a] *Laboratory of Biomolecular Chemistry, Institute of Biomedicinal Chemistry, Medical University, Lodz, Poland*
[b] *Laboratoire de Neuropsychopharmacologie Expérimentale, CNRS-FRE 2735, IFRMP 23, Université de Rouen, France*
[c] *Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland*
[d] *Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, Montreal, Canada*

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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.

* Corresponding author. Fax: +4842 6784277.
E-mail address: ajanecka@zdn.am.lodz.pl (A. Janecka).

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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