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Short communication

Characterization of endomorphin-1 and -2 on [^{35}S]GTPγS binding in the mouse spinal cord

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Abstract

In the present study, G-protein activation by newly-isolated opioid peptides, endomorphin-1 and -2, was examined in the mouse spinal cord by monitoring the binding of the non-hydrolyzable analog of GTP, guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS). Both endomorphin-1 and -2 increased [^{35}S]GTPγS binding to mouse spinal cord membranes in a concentration-dependent and saturable manner and reached a maximal stimulation of 57.3 ± 5.0 and 60.2 ± 3.2%, respectively, at 10 μM. In contrast, the synthetic selective μ-opioid receptor agonist [D-Ala^2,NHPhe^4,Gly-ol]enkephalin (DAMGO) had a much greater efficacy and produced 103.4 ± 5.4% of the maximal stimulation. The receptor specificity of endomorphin-stimulated [^{35}S]GTPγS binding was verified by co-incubating membranes with endomorphins in the presence of specific μ-(β-funaltrexamine and D-Phe-Cys-D-Tyr-Orn-Thr-Pen-Thr-NH_2 (CTOP)), δ-(naltrindole) or κ-(nor-binaltorphimine) opioid receptor antagonists. Co-incubation with either β-funaltrexamine or CTOP blocked both endomorphin-1- and -2-stimulated [^{35}S]GTPγS binding in a concentration-dependent manner, whereas neither naltrindole nor nor-binaltorphimine had any effect on the [^{35}S]GTPγS binding stimulated by either endomorphin-1 or -2. The data presented indicate that either endomorphin-1 or -2 activate G-proteins by specific stimulation of μ-opioid receptors, and may act as partial agonists with moderate catalytic efficacies in the mouse spinal cord. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Endomorphin; G-protein (Mouse)$_i$; Opioid peptide; μ-Opioid receptor; Spinal cord

1. Introduction

The recent cloning of μ-opioid receptors has indicated that the μ-opioid receptor has a seven-transmembrane topology, thus confirming that it belongs to the large superfamily of guanine nucleotide binding protein (G-protein)-coupled receptors (Chen et al., 1993; Liang et al., 1995). Agonist occupation of the μ-opioid receptor can lead directly to inhibition of adenylyl cyclase, activation of an inwardly rectifying K^+ conductance, and inhibition of a Ca^{2+} conductance through G-proteins (see the review by Law, 1995).

The G-proteins are heterotrimeric moieties composed of Gα-, Gβ- and Gγ-subunits. The agonist-stimulated coupling occurs via an exchange mechanism where GDP bound to Gα is exchanged for GTP in free solution. The binding of GTP produces a conformational change in the G-protein α-subunit, resulting in the dissociation of the receptor–G-protein complex and freeing of the α- and βγ-subunits to stimulate downstream events such as second messenger systems and ion channels. The α-subunit is spontaneously inactivated by an intrinsic GTPase, which hydrolyzes the GTP to GDP, ending the reaction of the G-protein cycle.

In an in vitro assay system, replacing the GTP with the non-hydrolyzable analogue, guanosine-5′-*O*-(3-[^{35}S]thio) triphosphate ([^{35}S]GTPγS), allows the measurement of the exchange process in the presence of added GDP. Unlike GTP, the addition of [^{35}S]GTPγS leads to the permanent formation of a Gα–[^{35}S]GTPγS complex, resulting in its accumulation in the membranes. Namely, if the receptor is coupled to G-proteins, the receptor agonist increases the amount of [^{35}S]GTPγS binding (Lorenzen et al., 1993;

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Sim et al., 1996). In our laboratory, the ability of opioid receptor agonist-stimulated [^{35}S]GTPγS binding has been reported in membranes prepared from the mouse brainstem and spinal cord (Mizoguchi et al., 1997; Narita et al., 1997; Narita and Tseng, 1998).

Opioid receptors are spontaneously activated by endogenous opioid peptides. The relatively large number of opioid peptides isolated in recent years is a reflection of the complexity of the endogenous opioid system. In 1997, Zadina et al. (1997) reported the isolation of two new μ-opioid receptor selective peptides from the mammalian brain. These peptides, which are called endomorphin-1 (Tyr–Pro–Trp–Phe–NH_2) and -2 (Tyr–Pro–Phe–Phe–NH_2), differ structurally from the previously described endogenous opioid peptides (such as the endorphins, enkephalins and dynorphins, which all have the amino-terminal amino-acid sequence Tyr–Gly–Gly–Phe) in their N-terminal (Tyr–Pro) sequence, C-terminal amidation and tetrapeptide length. Based on the receptor binding and behavioral assays, these peptides have been reported to show a high affinity and selectivity for the μ-opioid receptor in vivo (Zadina et al., 1997; Stone et al., 1997) and in vitro (Zadina et al., 1997) and thus may be specific endogenous ligands for the μ-opioid receptor. However, the functional activation of G-proteins induced by endomorphin-1 and -2 has not been clearly studied yet. The present study was then designed, for the first time, to investigate the effects of newly isolated opioid peptides, endomorphin-1 and -2, on [^{35}S]GTPγS binding to membranes from the mouse spinal cord.

2. Materials and methods

2.1. Animals

Male ICR mice weighing 23–30 g (SASCO, Omaha, NE) were used. Animals were housed five per cage in a room maintained at 22 ± 0.5°C with an alternating 12-h light–dark cycle. Food and water were available ad libitum. The animals were used only once.

2.2. Tissue preparation

The spinal cord was quickly excised on an ice-cold Petri dish. The spinal cord was homogenized in 15 vol. of ice-cold 0.32 M sucrose using a Potter–Elvehjem tissue grinder with a Teflon pestle. The homogenate was centrifuged $1000 \times g$ for 10 min and the supernatant was centrifuged at $20\,000 \times g$ for 20 min. The supernatant was discarded, and the pellet was resuspended in 15 vols. of 50-mM Tris–HCl (pH 7.4) and recentrifuged at $20\,000 \times g$ for 20 min. The resulting pellet was resuspended in a buffer (Buffer A) containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA and 100 mM NaCl, and stored at −70°C until used.

2.3. Binding assay

The suspensions were incubated at 25°C for 120 min in Buffer A with various concentrations of the agonist, 30-μM GDP and 50 pM [^{35}S]GTPγS (1000 Ci/mmol; Amersham, Arlington Heights, IL) in a total vol. of 1 ml. The reaction was terminated by filtering through Whatman GF/B glass filters, which had been previously soaked in 50 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$. The filters were then washed three times with 5 ml of Tris–HCl buffer (pH 7.4) at 4°C and transferred to scintillation counting vials. Then, 0.5 ml of Soluene-350 (Packard Instrument, Meriden, CT) and 4 ml of Hionic Fluor Cocktail (Packade Instrument) were added to the vials. After a 12-h equilibration period, the radioactivity in the samples was determined with a liquid scintillation analyzer (Model 1600 CA, Packade Instrument). Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Comparable results were obtained from more than three independent sets of experiments.

2.4. Drugs

The drugs used were: Tyr–Pro–Trp–Phe–NH_2 (endomorphin-1, provided by Toray Industries, Kamakura, Japan); Tyr–Pro–Phe–Phe–NH_2 (endomorphin-2, Toray Industries); [D-Ala^2,NHPhe^4,Gly-ol]enkephalin (DAMGO, Bachem California, Torrance, CA); β-funaltrexamine (β-FNA, Toray Industries); D-Phe-Cys-D-Tyr-Orn-Thr-Pen-Thr-NH_2 (CTOP, Bachem California), naltrindole hydrochloride (NTI, Toray Industries); nor-binaltorphimine (nor-BNI, Toray Industries); guanylyl-5′-*O*-(γ-thio)-triphosphate (GTPγS, Research Biochemicals International, Natick, MA); and guanosine-5′-*O*-(2-thio)-diphosphate (GDP, Sigma, St. Louis, MO).

2.5. Statistical analysis

The data are expressed as the mean ± S.E.M. The statistical significance of differences between the groups was assessed with the Newman–Keuls multiple comparison test.

3. Results

The conditions used in all subsequent experiments were 30–80 μg/ml of membrane protein in the presence of 30 μM GDP, 100 mM NaCl and 5 mM $MgCl_2$ at 25°C for 120 min. The concentration-effect curves for endomorphin-1- and -2-stimulated [^{35}S]GTPγS binding were examined in mouse spinal cord membranes. As shown in Fig. 1, endomorphin-1 increased [^{35}S]GTPγS binding in a concentration-dependent and saturable manner. The stimulatory effect of endomorphin-2 on [^{35}S]GTPγS binding was similar to endomorphin-1. Both endomorphin-1 and -2 reached

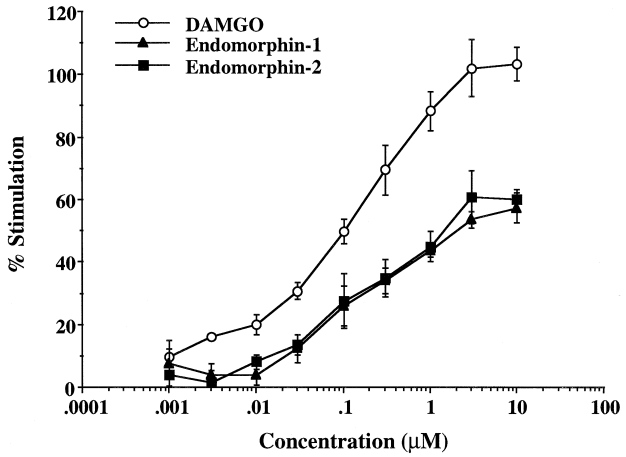

Fig. 1. [^{35}S]GTPγS binding in the mouse spinal cord. Membranes were incubated with 50 pM [^{35}S]GTPγS and 30 μM GDP with and without various concentrations of endomorphin-1 or -2 for 120 min at 25°C. The data are expressed as the percentage of basal [^{35}S]GTPγS (50 pM) binding measured in the presence of GDP and absence of agonist, and represent the mean ± S.E.M. from at least three separate experiments.

a maximal stimulation of 57.3 ± 5.0 and 60.2 ± 3.2%, respectively, at 10 μM. The synthetic selective μ-opioid receptor agonist DAMGO stimulated [^{35}S]GTPγS binding in a concentration-dependent manner and reached a maximal stimulation of 103.4 ± 5.4% at 10 μM. In addition, a classical type of μ-opioid receptor agonist morphine 10 μM stimulated [^{35}S]GTPγS binding maximally by 50.2 ± 6.6% over basal.

Since endomorphins are natural substances, experiments were also conducted in the presence of peptidase/protease inhibitors (10 μl/ml of a solution containing 0.2 mg/ml each of bestatin, leupeptin, pepstatin A and aprotinin). No significant differences in the [^{35}S]GTPγS binding stimulated by endomorphins or DAMGO were noted in the presence and absence of peptidase/protease inhibitors (58.9 ± 6.6, 62.8 ± 7.6 and 97.0 ± 2.9% of maximal stimulation for endomorphin-1, -2 and DAMGO at 10 μM, respectively, compared to peptidase/protease inhibitors alone).

In order to verify the specificity of this effect of the endomorphins, [^{35}S]GTPγS binding assay was conducted with endomorphins in the presence and absence of the specific μ-opioid receptor antagonists β-funaltrexamine and CTOP. As shown in Fig. 2, the increase of [^{35}S]GTPγS binding by either endomorphin-1 or -2 was blocked by co-incubation with β-funaltrexamine in a concentration-dependent manner. β-Funaltrexamine had no effect on the basal [^{35}S]GTPγS binding level when it was added alone. Either endomorphin-1- or -2-stimulated [^{35}S]GTPγS binding was also drastically attenuated by co-incubation with CTOP (10 μM endomorphin-1 plus 1- or 10 μM CTOP: 24.4 ± 5.6 or −1.4 ± 5.9% stimulation, respectively, $p <$ 0.01 vs. agonist alone; 10 μM endomorphin-2 plus 1- or 10 μM CTOP: 17.0 ± 2.1 or -2.6 ± 7.8% stimulation, respectively, $p < 0.01$ vs. agonist alone). The addition of 10 μM CTOP alone to the incubation solution had no effect on basal [^{35}S]GTPγS binding (−4.5 ± 3.4%).

In order to confirm the μ-opioid receptor specificity of endomorphin-stimulated [^{35}S]GTPγS binding, membranes were also incubated with the endomorphins in the presence and absence of selective δ-(naltrindole) or κ-(nor-binaltorphimine) opioid receptor antagonists. Concentrations of antagonist were chosen so that > 90% of the corresponding agonist-stimulated [^{35}S]GTPγS binding was selectively inhibited by the antagonist (Narita et al., 1997). Under these conditions, neither naltrindole nor nor-binaltorphimine had any effect on the stimulation of [^{35}S]GTPγS binding by either 10 μM endomorphin-1 or -2.

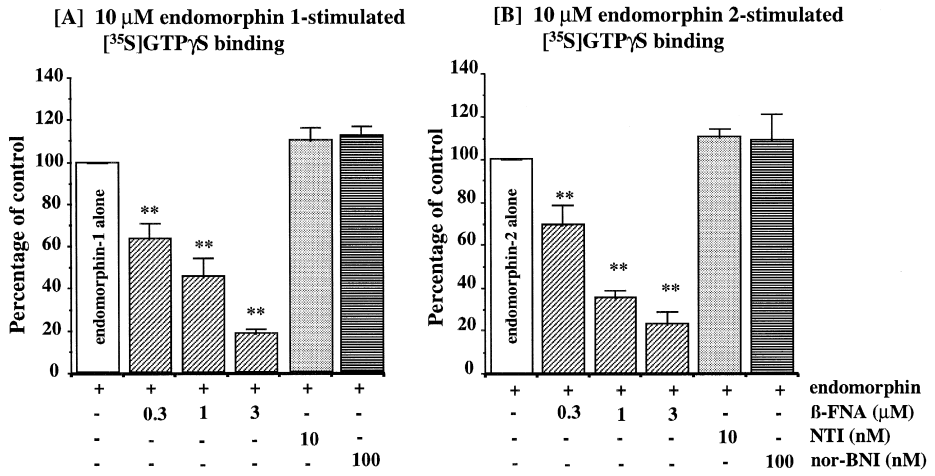

Fig. 2. Effect of the specific μ-(β-funaltrexamine), δ-(naltrindole) or κ-(nor-binaltorphimine) opioid receptor antagonists on [^{35}S]GTPγS binding stimulated by endomorphin-1 (A) or -2 (B) in the mouse spinal cord. Assays were performed in the presence of 50 pM [^{35}S]GTPγS, 30 μM GDP and 10 μM endomorphin-1 or -2 with and without β-funaltrexamine (β-FNA, 0.3–3 μM), naltrindole (NTI, 10 nM) or nor-binaltorphimine (nor-BNI, 100 nM). The data were expressed as the percentage of control and represent the mean ± S.E.M. from at least three separate experiments. ∗∗ $P < 0.01$ vs. agonist alone.

4. Discussion

It has been shown that the endomorphins have the highest specificity and affinity for the μ-opioid receptor of any endogenous substances found so far in the mammalian nervous system (Zadina et al., 1997). The studies of the distribution of endomorphin-1 by radioimmunoassay have indicated that endomorphin-1-like immunoreactivity is present in several brain areas known to also contain high concentrations of μ-opioid receptors and their mRNAs (Zadina et al., 1997; Mansour et al., 1995). More recent immunocytochemical studies have suggested that, like the distribution of the μ-opioid receptors in the spinal cord region (Arvidsson et al., 1995), a dense plexus of endomorphin-2 containing fibers is observed in the superficial dorsal horn of the spinal cord (Pierce et al., 1998). Although the cloning of the gene for the precursor pro-endomorphin should be studied, these findings provide evidence that the endomorphins may be natural selective ligands for the μ-opioid receptors.

The development of the opioid-stimulated [^{35}S]GTPγS binding assay in membranes has offered an opportunity to study the direct coupling of opioid receptors to the activation of G-proteins and to determine the efficacy and intrinsic activity of opioid agonists to activate G-proteins (Traynor and Nahorski, 1995; Sim et al., 1996; Selley et al., 1997). The present study was designed to use this technique for the first time to investigate the effects of newly isolated opioid peptides, endomorphin-1 and -2, to activate G-proteins in the spinal cord of the mouse. We found that both endomorphin-1 and -2 were able to stimulate the binding of [^{35}S]GTPγS to mouse spinal cord membranes in a concentration-dependent and saturable manner with similar efficacies. The effects of the endomorphins were reversed by the addition of the selective μ-opioid receptor antagonists CTOP and β-funaltrexamine. CTOP is a highly-selective μ-opioid receptor antagonist, whereas β-funaltrexamine may have a reversible κ-opioid receptor agonistic activity as well as a long-lasting μ-opioid receptor antagonistic property (Qi et al., 1990). Under the present condition, β-funaltrexamine had no effect on the basal [^{35}S]GTPγS binding level when it was added alone. In addition, the selective κ-opioid receptor agonist, *trans*(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] (U50,488H), did not antagonize the endomorphin-stimulated [^{35}S]GTPγS binding (unpublished observation). These findings indicate that the attenuation of endomorphin-stimulated [^{35}S]GTPγS binding by β-funaltrexamine presented is due to the blockade of μ-opioid receptors. Taken together, the results presented clearly suggest that G-protein activation stimulated by both endomorphin-1- and -2 is a μ-opioid receptor-mediated event.

On the contrary, both naltrindole at 10 nM and nor-binaltorphimine at 100 nM, at doses that completely blocked selective δ-opioid receptor agonist [D-Pen^{2,5}]enkephalin- and U50,488H-stimulated [^{35}S]GTPγS binding to mouse spinal cord membranes, respectively (Narita et al., 1997), had no effect on either endomorphin-1- or -2-stimulated [^{35}S]GTPγS binding, thus confirming the μ-opioid receptor specificity of these effects.

It has been reported that different μ-selective opioid receptor agonists have different efficacies in stimulating [^{35}S]GTPγS binding to membranes prepared from human neuroblastoma SH-SY5Y cells (Traynor and Nahorski, 1995), rat thalamus (Selley et al., 1997), human neuroblastoma SK-N-SH cells (Selley et al., 1997), and CHO cells transfected with the cloned mouse μ-opioid receptor (Selley et al., 1997), as measured by the different maximal stimulation of [^{35}S]GTPγS binding in agonist concentration-effect curves. The μ-opioid receptor agonists, morphine and fentanyl, produced 55 to 60% of the maximal stimulation observed with DAMGO in rat thalamus and human neuroblastoma SK-N-SH cells (Selley et al., 1997). Buprenorphine was much less efficacious and produced only 10% of the maximal stimulation observed with DAMGO in rat thalamus and human neuroblastoma SK-N-SH cells (Selley et al., 1997). It is most likely that these differences are due to the different intrinsic efficacies of these agonists to activate G-proteins through μ-opioid receptors. In the present study, maximally effective concentrations of endomorphins (10 μM) stimulated [^{35}S]GTPγS binding by 55 to 58% of the magnitude observed with maximally effective concentrations of DAMGO. These results indicate that endomorphins may belong to the same class to morphine in the mouse spinal cord with regard to its efficacy for G-protein activation. Furthermore, we could exclude the possibility that the comparable lower levels of endomorphin-stimulated [^{35}S]GTPγS binding may result from the quick degradation of endomorphins by intrinsic enzymes, because no differences in the [^{35}S]GTPγS binding stimulated by endomorphins were noted in the presence and absence of peptidase/protease inhibitors. These findings clearly suggest that both endomorphin-1 and -2 may act as partial μ-opioid receptor agonists in the mouse spinal cord.

In conclusion, the data presented indicate that both endomorphin-1 and -2 stimulate G-proteins by the specific activation of μ-opioid receptors. Although a more elaborate analysis of distribution of endomorphin-containing fibers and μ-opioid receptor ligand-binding studies should be undertaken, it is most likely that both endomorphin-1 and -2 may act in the mouse spinal cord as endogenous partial μ-opioid receptor agonists with moderate catalytic activity.

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