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Short communication

Endomorphin-1 and endomorphin-2 are partial agonists at the human μ-opioid receptor

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Abstract

Recently two tetrapeptide ligands that bind preferentially to the μ-opioid receptor were identified and named endomorphin-1 and endomorphin-2. We examined the ability of these peptides to stimulate G protein activation in human μ-opioid receptor transfected B82 fibroblasts as measured by [^{35}S]GTPγS binding to cell membranes. Both endomorphin-1 and -2 act as partial agonists in this assay system compared with the μ-selective agonist [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]enkephalin (DAMGO). In addition, endomorphins demonstrate efficacy similar to morphine. These findings demonstrate that endomorphin peptides have similar activity at the μ-opioid receptor as morphine and suggest that these peptides have the potential to modulate neuronal activity in vivo. © 1998 Elsevier Science B.V.

Keywords: μ-Opioid receptor; Endomorphin-1; Endomorphin-2; Efficacy

1. Introduction

Over 20 years ago, Kosterlitz and his coworkers began to investigate the purpose of opioid receptors in vivo (Hughes et al., 1975; Lord et al., 1977). These investigators reasoned that since opioid receptors exist, there must also be endogenous ligands that interact with these receptors. Concurrently with this work it was demonstrated that there are three different opioid receptors, namely the δ-, κ- and μ-opioid receptors. These receptors have been renamed as OP_1, OP_2 and OP_3 receptors, respectively, based on the order in which these G protein coupled receptors were cloned (Dhawan et al., 1996). Endogenous peptide ligands that activate opioid receptors have previously been identified (Brownstein, 1993; Hughes et al., 1975); however, a new class of μ-opioid receptor-selective peptides have only recently been isolated from the mammalian brain (Zadina et al., 1997). These peptides, Tyr-Pro-Trp-Phe-NH_2 and Tyr-Pro-Phe-Phe-NH_2, are known as endomorphin-1 and endomorphin-2, respectively. Endomorphin-1 is 4000- and 15 000-fold selective for μ-opioid receptors as compared to δ- and κ-opioid receptors, respectively, whereas endomorphin-2 is $>$ 13 000- and $>$ 7500-fold selective, respectively. Both peptides mediate analgesia in mice after intracerebroventricular injection with similar potency to morphine (Zadina et al., 1997) as well as mediate analgesia after intrathecal injection (Stone et al., 1997). These peptides also have been demonstrated to have hypotensive activity in rabbits (Champion et al., 1997).

The EC_{50} (potency) of a drug in a functional assay is dictated by: (a) the affinity of drug for receptor; (b) the receptor density in a test tissue or cell; and (c) the capacity of the drug bound receptor to initiate a functional response (Ruffolo, 1982). In contrast, efficacy is a measure of the coupling efficiency of drug-bound receptors to activate a functional response. Efficacy is calculated utilizing the potency value in a functional assay and the inhibition constant (K_i) determined from the displacement of radiolabeled antagonist from μ-opioid receptors by drug (Ehlert, 1985). While observed efficacy values are dependent on the receptor density in the test tissue (Furchgott, 1966), we believe that efficacy values better characterize the ability of drugs to stimulate a functional response as compared to potency values. In this report we have determined the efficacy of the newly discovered endogenous μ-opioid receptor agonists endomorphin-1 and endomorphin-2 to

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modulate the first step in opioid-mediated signal transduction, namely G protein activation.

2. Materials and methods

2.1. Preparation of membranes from human μ-opioid receptor transfected B82 fibroblasts

Stably transfected B82 fibroblasts, expressing the human μ-opioid receptor at 151 fmol/mg membrane protein, were previously prepared (Knapp et al., 1995). The cell line was grown in 162 cm^2 culture flasks in Dulbecco's Modified Eagles Medium: Nutrient Mixture F12 with 5% fetal calf serum, 5% newborn calf serum, G418 (500 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). Growth medium was removed when the cells were 80% confluent and cells were removed from the plate by a 5-min incubation in phosphate buffered saline containing 0.02% EDTA. Cells were then sedimented at 1500 × *g* and homogenized in ice cold Tris (10 mM)/EDTA (1 mM) buffer with a dounce homogenizer (10 strokes). After sedimentation (40 000 × *g*, 10 min) membranes were homogenized as above in assay buffer (25 mM Tris–HCl, 150 mM NaCl, 2.5 mM $MgCl_2$, 1.0 mM EDTA, 50 μM GDP, 30 μM bestatin, 10 μM captopril and 0.1 mM phenylmethylsulfonyl fluoride, pH = 7.4) and sedimented as above. Membranes were then resuspended to OD_{280} = 3.0 in assay buffer. This stock solution was then diluted 10-fold (final OD_{280} = 0.3) for the binding assays described below.

2.2. Determination of agonist K_i values

The K_i values of [D-Ala^2,*N*-Me-Phe^4, Gly-ol^5]enkephalin (DAMGO), morphine sulfate (both from RBI, Natick, MA), and endomorphin-1 and -2 (both synthesized in the laboratory of Victor Hruby) were determined using cell membranes prepared from human μ-opioid receptor-transfected B82 cells. IC_{50} values were determined by competitive binding of drugs against the opioid receptor antagonist [^{3}H]diprenorphine for 90 min at 30°C in assay buffer. Bound [^{3}H]diprenorphine (0.4 nM, Amersham, Arlington Heights, IL; 39 Ci/mmol) was separated from unbound by rapid vacuum filtration through GF/B glass fiber filters (Whatman, Gaithersburg, MD) followed by four washes with ice-cold 25 mM Tris/120 mM NaCl, pH = 7.4. Filters were pretreated with 0.05% polyethylenimine to reduce background binding. IC_{50} values were corrected to K_i values using the Cheng–Prusoff equation (Cheng and Prusoff, 1973) with the K_d of [^{3}H]diprenorphine previously determined to be 0.22 nM in assay buffer (data not shown). Data were analyzed using a single site competition model with Prism® Version 2 (GraphPad, San Diego, CA).

2.3. Determination of G protein activation by [^{35}S]GTPγS binding

G protein activation can be measured as drug-stimulated [^{35}S]GTPγS binding to membranes (Lorenzen et al., 1993; Traynor and Nahorski, 1995; Sim et al., 1996) and will be performed as previously described (Quock et al., 1997) to determine endomorphin-1-, endomorphin-2-, morphine- and DAMGO-mediated activation of G proteins. Briefly, membranes from human μ-opioid receptor transfected B82 cells were incubated in assay buffer in the presence of 0.1 nM [^{35}S]GTPγS (1250 Ci/mmol, DuPont New England Nuclear, Boston, MA) with increasing concentrations of drug for 90 min at 30°C. Bound radiolabel was then separated from unbound [^{35}S]GTPγS by vacuum filtration as above after glass fiber filters were pretreated with assay buffer. Data from [^{35}S]GTPγS binding assays were analyzed using a fixed slope (Hill slope = 1) sigmoidal dose response curve with Prism® Version 2.

2.4. Efficacy calculations

The K_i and the EC_{50} and maximal response from [^{35}S]GTPγS binding assays were used to calculate the efficacy of drug activity using the equation: efficacy = $E_{max-A}/E_{max} \times (K_i/EC_{50} + 1) \times 1/2$ where E_{max-A} is the maximal response elicited by the test agonist and E_{max} is the maximal response elicited by the agonist stimulating the greatest functional response in the system (Ehlert, 1985). The maximum [^{35}S]GTPγS binding response elicited by DAMGO was taken as the E_{max} in these experiments.

2.5. Statistical analysis

Differences between the calculated K_i and EC_{50} values for each drug were examined for statistical significance utilizing an unpaired 2 tailed *t*-test. Differences between the E_{max} values (intrinsic activity) and EC_{50} values (potency) were analyzed by Analysis of Variance and significant differences between treatment groups determined with a Neuman–Keuls multiple range test.

3. Results

We initially examined G protein activation stimulated by the μ-opioid receptor-selective agonists DAMGO, morphine, endomorphin-1 and endomorphin-2 as measured by [^{35}S]GTPγS binding to cell membranes (Fig. 1A). The rank order for maximal stimulation (intrinsic activity) was DAMGO > endomorphin-2 ≈ endomorphin-1 = morphine. The intrinsic activity of DAMGO was significantly greater than that of the other μ-opioid receptor selective agonists. Differences in intrinsic activity between endomorphin-1, endomorphin-2 and morphine were insignificant. Conversely, we observed the following rank order of potency

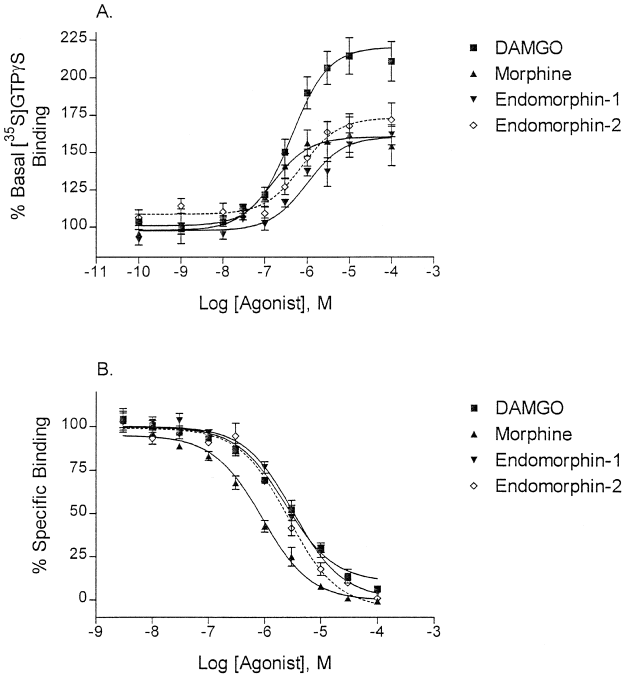

Fig. 1. (A) G protein activation as determined by [^{35}S]GTPγS binding. Membranes prepared from human μ-opioid receptor transfected B82 cells were incubated with increasing concentrations of agonist and 0.1 nM [^{35}S]GTPγS. The average value for 100% basal [^{35}S]GTPγS binding, corresponding to [^{35}S]GTPγS binding to membranes in the absence of agonist, was 1915, 1834, 1642 and 1731 CPM for endomorphin-1, endomorphin-2, morphine and DAMGO concentration response curves, respectively. Assay tubes contained 50 μg protein as determined with the Lowry Protein Reagent (Sigma). (B) Inhibition of [^{3}H]-diprenorphine (0.4 nM) binding to membranes prepared from human μ-opioid receptor transfected B82 cells by increasing concentrations of agonist. Specific binding is defined as agonist displaceable counts with 100% equal to specific [^{3}H]diprenorphine binding in the absence of agonist. $N = 3$ for most experiments. $n = 5$ for [^{35}S]GTPγS and competition binding experiments using DAMGO.

for these drugs: morphine > DAMGO > endomorphin-2 ≈ endomorphin-1; however, the only significant difference in potency within this set of drugs was between morphine and endomorphin-1 ($P < 0.05$).

We next determined the K_i of these drugs from the IC_{50} against the antagonist [^{3}H]diprenorphine in competition binding assays (Fig. 1B). All drugs tested completely inhibited [^{3}H]diprenorphine binding and the concentration response curves of drugs fit closely to a curve with a Hill Slope = 1. These experiments were performed in GTPγS assay buffer in the presence of 150 mM NaCl and 50 μM GDP as it is an assumption of the efficacy calculations that binding assays are conducted under the same experimental conditions as the functional assay ([^{35}S]GTPγS binding). The rank order of affinity for these agonists was morphine > DAMGO ≈ endomorphin-2 ≈ endomorphin-1. The K_i values that we calculated for DAMGO and morphine are consistent with those previously reported in buffer containing 100 mM NaCl and 50 μM GDP (Emmerson et al., 1996).

Data presented in Fig. 1 were then utilized to calculate the efficacy of μ-opioid selective drugs with the equation efficacy $= E_{max-A}/E_{max} \times (K_i/EC_{50} + 1) \times 1/2$ (Table 1). In contrast to the potencies, the following rank order of efficacy was observed: DAMGO > morphine > endomorphin-2 ≈ endomorphin-1.

4. Discussion

Efficacy values describe the relationship between drug occupancy of receptors and the stimulation of a functional response. When comparing efficacy values for two drugs that bind to the same receptor, the drug with the higher efficacy value will occupy a smaller fraction of available receptor to mediate a given level of functional response. When efficacy is calculated according to the methods utilized in this paper, full agonists have efficacies of one or greater. When observed efficacy values are greater than one, more receptors are present on the cell or tissue than are necessary to yield a maximal response (spare receptors). Partial agonists have efficacy values of less than one. On initial inspection of Table 1, this does not appear to hold true for morphine. However, in contrast to the data for DAMGO, the differences between the K_i and EC_{50} values were not significantly different for morphine, endomorphin-1 and endomorphin-2. If the K_i/EC_{50} ratio in the efficacy equation is held to unity for morphine, endomorphin-1 and endomorphin-2 to reflect this lack of significance, then the calculated efficacy values for these drugs are 0.73, 0.73 and 0.79, respectively. Thus, in this system morphine, endomorphin-1 and endomorphin-2 are equally efficacious and are partial agonists.

Table 1
The efficacies of μ-opioid receptor-selective drugs

	EC_{50} (nM)	E_{max} (%)	K_i (nM)	Efficacy	Relative efficacy
DAMGO	423 ± 56	220 ± 12	800 ± 102	1.45	1
Morphine	147 ± 36	160 ± 12	305 ± 58	1.12	0.77
Endomorphin-1	975 ± 348	160 ± 7.1	1117 ± 205	0.78	0.54
Endomorphin-2	753 ± 61	174 ± 9.8	900 ± 67	0.86	0.59

K_i values were determined from [^{3}H]diprenorphine competition binding experiments and EC_{50} values from [^{35}S]GTPγS binding experiments.
% Stimulation represents the maximal stimulation of [^{35}S]GTPγS binding over basal as calculated by Prism® Version 2.
K_i, EC_{50} and E_{max} values represent the mean ± S.E.M.

Initial studies of endomorphin-1 and endomorphin-2 reported high affinity values of 0.36 and 0.69 nM, respectively (Zadina et al., 1997). In contrast we observed K_i values of 1117 and 900 nM for endomorphin-1 and endomorphin-2, respectively. The lower affinity of these peptides in our studies is most likely due to the high sodium (150 mM) and GDP (50 μM) concentrations found in the [^{35}S]GTPγS binding assay (Rosenberger et al., 1980) as (1) the original studies of Zadina were performed in the absence of sodium (Zadina et al., 1994, 1997) and (2) the K_i values of 800 and 305 nM that we report for DAMGO and morphine are in reasonable agreement with K_i values previously reported for these drugs in the presence of sodium (Emmerson et al., 1996). We verified this explanation by performing competition binding experiments in assay buffer in which NaCl and GDP were omitted with [^{3}H]diprenorphine as the antagonist. High affinity K_i values of 4.0, 7.7 and 5.9 nM were observed for DAMGO, endomorphin-1 and endomorphin-2, respectively (data not shown).

We have found that endomorphin-1 and endomorphin-2 are partial agonists in membranes isolated from human μ-opioid receptor transfected B82 cells as DAMGO stimulates significantly more G protein activation as compared to morphine and endomorphins. Endomorphin 1 and 2 are equally efficacious as morphine. These findings indicate that the endogenous peptides endomorphin-1 and endomorphin-2 have the potential to modulate brain function through μ-opioid receptors provided these agonists are present in sufficient concentration.

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