Peptides 55 (2014) 145–150

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Pharmacological characterization of endomorphin-2-based cyclic pentapeptides with methylated phenylalanine residues

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ARTICLE INFO

Article history:
Received 29 January 2014
Received in revised form 25 February 2014
Accepted 2 March 2014
Available online 13 March 2014

Keywords:
Solid phase peptide synthesis
Cyclic peptides
Opioid receptors
Receptor binding
Calcium mobilization
Hot-plate test

ABSTRACT

As part of our continuing studies on the structure–activity relationships of cyclic pentapeptides based on the structure of endomorphin-2, we report here the synthesis and biological activities of a new series of analogs incorporating 2′, 3′ or 4′-methylphenylalanine (MePhe) residues into positions 3 or 4 of the parent cyclopeptide, Dmt-c[D-Lys-Phe-Phe-Asp]NH_2 (Dmt = 2′,6′-dimethyltyrosine). Analogs with MePhe in position 4 showed a row of magnitude increased μ-opioid receptor (MOP receptor) affinity as compared with a parent compound. The in vitro potencies of the new analogs were determined in calcium mobilization assay performed in Chinese Hamster Ovary (CHO) cells expressing human recombinant opioid receptors and chimeric G proteins. All analogs were strong μ/κ (MOP/KOP) receptor agonists and weak δ (DOP) receptor agonists. In the in vivo hot-plate test in mice, the $MePhe^4$-modified peptides showed remarkable antinociceptive activity after intracerebroventricular (i.c.v.) administration which was most likely due to the concomitant activation of more than one opioid receptor type.

1. Introduction

Opioid receptors belong to the family of the G-protein-coupled receptors and are categorized into so called classical opioid receptors: μ (MOP), δ (DOP), κ (KOP) and nociceptin/orphanin (NOP) receptor which shares high sequence similarity with MOP, DOP and KOP receptors but has a markedly distinct pharmacology [12]. Classical opioid receptors are found in the central and peripheral nervous system, where they play a crucial role in mediation of analgesia. Since in 1975 Hughes et al. [11] discovered the two pentapeptides (Met-enkephalin and Leu-enkephalin) which acted as agonists at the opioid receptor sites, further exploration in many laboratories over the last 40 years led to the identification of endogenous ligands for all receptor types [12,14]. Numerous synthetic analogs of opioid peptides have also been synthesized and extensively studied as potential painkillers. The strongest analgesic effects are mediated by the MOP receptor [31], and most of the currently available therapeutic non-peptide agents for pain relief, such as morphine and its derivatives, show some degree of MOP selectivity. However, the chronic use of such drugs is associated with serious side effects, including constipation, respiratory depression, tolerance, and dependence. A major challenge for the chemists is to improve pharmacological profile of new analogs and to minimize their adverse effects. A possible solution may lie in achieving ligands that simultaneously bind to more than one opioid receptor type [5,26,29]. For example, compounds with MOP agonist/DOP antagonist profile show greatly reduced potential to produce tolerance and dependence [1,5,30], while DOP agonists can enhance the analgesic potency of MOP agonists [15,27,36]. KOP agonists are known to produce analgesia accompanied by some dysphoric effects which hindered their development for human use. However, the advantageous effect of KOP agonists for the treatment of

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http://dx.doi.org/10.1016/j.peptides.2014.03.001
0196-9781/

cocaine abuse has been documented [21]. So far not much is known about the analgesic action of compounds with a mixed MOP/KOP agonist profile. Quite recently published article of Greedy et al. [9] describes non-peptide ligands with such properties.

In the opioid peptides, cyclization of linear sequences has often been shown to result in the drastic changes in the affinity to opioid receptors [25]. For some time now our group [7,13,23,24] has been focused on synthesizing cyclic analogs based on the structure of the endogenous MOP receptor-selective ligand, endomorphin-2 (EM-2: Tyr-Pro-Phe-Phe-NH_2) [34]. Here, using the previously described structure Dmt-c[D-Lys-Phe-Phe-Asp]NH_2 [7] as a parent compound, we synthesized a series of analogs in which Phe^3 and Phe^4 residues were consecutively replaced by 2′-, 3′- or 4′-MePhe.

These compounds were pharmacologically characterized in vitro in receptor binding and functional assays and evaluated in vivo in the mouse hot-plate test.

2. Materials and methods

2.1. Solid-phase peptide synthesis

Methylated phenylalanines were purchased from TriMen Chemicals Ltd., Lodz, Poland. All peptides were synthesized as previously described [24] using techniques for 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids on MBHA Rink-Amide peptide resin (100–200 mesh, 0.8 mM/g, Novabiochem). Dmt hydroxyl was protected by the *t*-Bu group and the hyper-acid labile Mtt/O-2Ph*i*Pr groups were used for the selective D-Lys and Asp side-chain protection.

Crude peptides were purified by semi-preparative RP-HPLC using Waters Breeze instrument (Milford, MA, USA). The purity of the final products was verified by analytical RP-HPLC and mass spectrometry using Bruker Apex Ultra 7T FT-ICR mass spectrometer (Billerica, MA, USA) with electrospray ionization (ESI-MS).

2.2. Animals

The procedures used in this study were approved by the Local Ethical Committee for Animal Research with the following numbers: 78/LB 641/2012 (rats) or N/10-04-04-12 and N/12-04-04-14 (mice).

Male Wistar rats (S3, Animal House, Faculty of Pharmacy, Lodz, Poland), weighing 200–250 g were used as a source of brain membranes and Male Swiss albino mice (CD1, JANVIER LABS, Le Genest-Saint-Isle, France), weighing 20–26 g, were used in the in vivo experiments. Animals were housed at a constant temperature (22 ± 1 °C) and maintained under a 12-h light/dark cycle in sawdust coated plastic cages with access to standard laboratory chow and tap water ad libitum.

2.3. Opioid receptor binding assays

Opioid receptor binding studies were performed according to the method described in detail elsewhere [23]. Binding affinities for MOP and DOP receptors were determined by displacing, respectively, [^{3}H]-DAMGO and [^{3}H][$Ile^{5,6}$]-deltorphin-2 from Wistar rat brain membrane binding sites. Three independent experiments for each assay were carried out in duplicate. The data were analyzed by a nonlinear least square regression analysis computer program Graph Pad PRISM 4.0 (GraphPad Software Inc., San Diego, USA).

2.4. Calcium mobilization assay

Chinese Hamster Ovary (CHO) cells stably co-expressing human recombinant KOP or MOP receptors and the C-terminally modified $G\alpha_{qi5}$ [4], and CHO cells co-expressing the human recombinant DOP receptor and the $G\alpha_{qG66Di5}$ [17] chimeric protein were generated as previously described [2,3]. Cells were cultured in Dulbecco's MEM/HAMS F12 (1:1) culture medium supplemented with 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 mg/ml), geneticin (G418; 200 μg/ml) and hygromycin B (100 μg/ml). Cell cultures were kept at 37 °C in 5% CO_2/humidified air. When confluence was reached (3–4 days), cells were sub-cultured using trypsin/EDTA and used for the assay.

Cells were seeded at a density of 50,000 cells/well into 96-well black, clear-bottom plates. After 24 h incubation, the cells were treated with the loading solution of the culture medium supplemented with 2.5 mM probenecid, 3 μM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid for 30 min at 37 °C. The loading solution was aspirated and a 100 μl/well of the assay buffer (Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES, 2.5 mM probenecid, and 500 μM Brilliant Black) was added.

The tested peptides were dissolved in the bi-distilled water to the final concentration of 1 mM. The successive dilutions were made in the HBSS/HEPES (20 mM) buffer (containing 0.005% BSA fraction V).

After placing both plates (cell culture and compound plate) into the FlexStation II, the on-line additions were carried out in a volume of 50 μl/well and fluorescence changes were measured at 37 °C.

Agonist potencies were given as pEC_{50} representing a negative logarithm of the molar concentration of an agonist that produces 50% of the maximal possible effect. Concentration response curves were fitted with the four parameter logistic nonlinear regression model:

$$\text{Effect} = \text{baseline} + \frac{E_{\max} - \text{baseline}}{1 + 10^{(\log EC_{50} - X)\cdot n}}$$

where X is the agonist concentration and n is the Hill coefficient. Ligand efficacy was expressed as intrinsic activity (α) calculated as the $E_{\max}$ of the ligand to $E_{\max}$ of the standard agonist ratio.

At least three independent experiments for each assay were carried out in duplicate. Curve fittings were performed using Graph Pad PRISM 5.0 (GraphPad Software Inc., San Diego, USA). Data have been statistically analyzed with one way ANOVA followed by the Dunnett's test for multiple comparisons; p values <0.05 were considered to be significant.

2.5. Metabolic stability

Enzymatic degradation studies of the new analogs were performed using the Wistar rat brain homogenate, following a method reported in detail previously [23]. Briefly, the analogs were incubated with brain homogenate over 0, 7.5, 15, 22.5, 30 and 90 min at 37 °C. The reaction was monitored by RP-HPLC and the amount of the remaining peptide (area %) was assessed.

Three independent experiments for each assay were carried out in duplicate. The data have been statistically analyzed with one way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. A probability level of 0.05 or smaller was used to indicate statistical significance.

2.6. Assessment of antinociception

The procedures used in this study were in accordance with the European Communities Council Directive of 24 November 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.

The analgesic activity of peptides was assessed in the hot-plate test in mice after i.c.v. administration, as described earlier [7]. All compounds were dissolved in DMSO (0.1%, v/v) and diluted in