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Structural and pharmacological characteristics of chimeric peptides derived from peptide E and β-endorphin reveal the crucial role of the C-terminal YGGFL and YKKGE motifs in their analgesic properties

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ABSTRACT

Peptide E (a 25-amino acid peptide derived from proenkephalin A) and β-endorphin (a 31-amino acid peptide derived from proopiomelanocortin) bind with high affinity to opioid receptors and share structural similarities but induce analgesic effects of very different intensity. Indeed, whereas they possess the same N-terminus Met-enkephalin message sequence linked to a helix by a flexible spacer and a C-terminal part in random coil conformation, in contrast with peptide E, β-endorphin produces a profound analgesia. To determine the key structural elements explaining this very divergent opioid activity, we have compared the structural and pharmacological characteristics of several chimeric peptides derived from peptide E and β-endorphin. Structures were obtained under the same experimental conditions using circular dichroism, computational estimation of helical content and/or nuclear magnetic resonance spectroscopy (NMR) and NMR-restrained molecular modeling. The hot-plate and writhing tests were used in mice to evaluate the antinociceptive effects of the peptides. Our results indicate that neither the length nor the physicochemical profile of the spacer plays a fundamental role in analgesia. On the other hand, while the functional importance of the helix cannot be excluded, the last 5 residues in the C-terminal part seem to be crucial for the expression or absence of the analgesic activity of these peptides. These data raise the question of the true function of peptides E in opioidergic systems.

1. Introduction

Mammalian opioid peptides are a family of neuropeptides that are processed from at least four large precursor proteins known as proopiomelanocortin, proenkephalin A, prodynorphin and pronociceptin/orphanin-FQ [15,25]. *Except for those derived from* pronociceptin/orphanin-FQ, all endogenous opioid peptides derived from the three other precursors share a common N-terminal sequence (Leu-enkephalin YGGFL or Met-enkephalin YGGFM), which interacts with specific membrane receptors termed μ, δ and κ opioid receptors [29,59]. Peptide E is a 25-amino acid opioid peptide derived from proenkephalin A that was originally isolated from the bovine adrenal medulla [30]. Bovine peptide E (BPE) has the particularity to possess a Met-enkephalin and a Leu-enkephalin sequence at its N- and C-terminus, respectively (Fig. 1). Since its discovery, several in vivo and in vitro pharmacological studies suggested that BPE is a potent opioid peptide, which produces substantial analgesia after intracerebroventricular injection [16,17,24,30].

Fifteen years after the first description of BPE, its amphibian homologue (FPE) has been isolated from the brain of the European green frog *Rana ridibunda* [14]. Considering their primary structures (Fig. 1), FPE was expected to be as analgesic as BPE. Indeed, in addition to their very similar primary structures (FBE differs from BPE by only two substitutions, $M^{15} \rightarrow Q^{15}$ and $L^{25} \rightarrow M^{25}$), these peptides possess at their N-terminus the same sequence (Met-enkephalin) that is known to be necessary for interaction with the receptor-binding pocket [1,19,26]. Thus, using as a reference β-endorphin, an endogenous opioid peptide known for its potent and sustained analgesic effects [21,40], a structure–activity relationship study has been carried out on BPE and FPE [13]. Paradoxically, the hot-plate and acetic acid-induced writhing tests showed that, comparatively to β-endorphin, peptides E are

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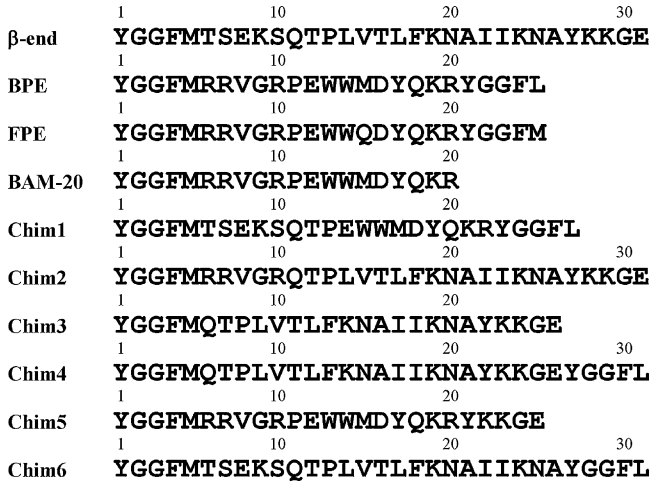

Fig. 1. Sequences of β-endorphin, bovine peptide E (BPE), frog peptide E (FPE), bovine adrenal medulla peptide 20 (BAM-20) and chimeric peptides Chim1, Chim2, Chim3, Chim4, Chim5 and Chim6.

virtually devoid of opioid activity. Furthermore, NMR-restrained molecular modeling calculations revealed that the structures of FPE and BPE are very similar [12]. Taken together, these results demonstrated that the two substitutions which differentiate BPE from FPE do not seem important for their secondary structure and their analgesic property.

Several studies on ligand-receptor interactions indicate that the structure of opioid peptides can be divided into two domains that are functionally distinct: a common "message" domain, which confers bioactivity, and a variable "address" domain, which controls most of the selectivity [51,54–58]. Peptides E and β-endorphin share the same N-terminus Met-enkephalin message moiety and, in spite of their very different primary structures (Fig. 1), these peptides were shown to adopt a common central structure surrounded with flexible segments [12,13,39,53,66]. Thus, although these peptides seem to have similar structure components, some slight structural variations in their address domain could significantly influence the peptide–receptor interactions and thus induce differential effects on nociception.

In order to identify the structural elements that can account for the very divergent opioid activity of these two peptides, we have investigated the structure–activity relationships of BPE, β-endorphin and various chimeric peptides. The structures were compared under the same experimental conditions using circular dichroism (CD), computational estimation of helical content and/or nuclear magnetic resonance spectroscopy (NMR) and NMR-restrained molecular modeling. The antinociceptive activities were evaluated using the hot-plate and acetic acid-induced writhing tests. The role of the flexible segment separating the Met-enkephalin sequence from the central helix (the spacer) was examined by creating chimeric peptides Chim1, Chim2 and Chim3 which correspond respectively to BPE with the spacer of β-endorphin, β-endorphin with the spacer of BPE and β-endorphin without spacer (Fig. 1). Chim4, Chim5 and Chim6 were designed to test the influence of the five C-terminal residues. Chim4 was obtained by adding a Leu-enkephalin sequence at the C-terminus of Chim3 whereas Chim5 and Chim6 were created by inverting the C-terminal last five residues of β-endorphin and BPE (Fig. 1).

2. Materials and methods

2.1. Drugs, chemicals and reagents

Synthetic human β-endorphin (YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE) was purchased from Neosystem (Strasbourg, France). L-Amino acid residues were from Senn Chemicals (Dielsdorf, Switzerland). The preloaded 4-hydroxymethyl-phenoxymethyl-copolystyrene-1%-divinylbenzene (HMP) resins, O-benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxy-benzotriazole (HOBt) were from Applera-France (Courtaboeuf, France). Acetonitrile and N-methylpyrrolidinone (NMP) were from Carlo Erba (Val-de-Reuil, France). Trifluoroethanol (TFE), NMR-grade methanol-d3 (MeOH-d3) and tetramethylsilane (TMS) were from Euriso-Top (Gif-sur-Yvette, France). Diisopropylethylamine (DIEA), piperidine, trifluoroacetic acid (TFA), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), sodium dodecyl sulphate (SDS) and other reagents were from Sigma–Aldrich (Saint-Quentin-Fallavier, France).

2.2. Peptide synthesis

BPE (YGGFMRRVGRPEWWMDYQKRYGGFL), BAM-20 (YGGFMRRVGRPEWWMDYQKR), Chim1 (YGGFMTSEKSQTPEWWMDYQKRYGGFL), Chim2 (YGGFMRRVGRQTPLVTLFKNAIIKNAYKKGE), Chim3 (YGGFMQTPLVTLFKNAIIKNAYKKGE), Chim4 (YGGFMQTPLVTLFKNAIIKNAYKKGEYGGFL), Chim5 (YGGFMRRVGRPEWWMDYQKRYKKGE) and Chim6 (YGGFMTSEKSQTPLVTLFKNAIIKNAYGGFL) were synthesized (0.1 mmol scale) on a Fmoc-Leu-HMP resin (BPE, Chim1, Chim4 and Chim6), Fmoc-Glu(OtBu)-HMP resin (Chim2, Chim3 and Chim5) or Fmoc-Arg(Pbf)-HMP resin using an Applied Biosystems model 433A automatic peptide synthesizer and the standard procedures, as previously described [35,36]. All Fmoc-amino acids (1 mmol, 10 equiv.) were coupled by *in situ* activation with HBTU/HOBt (1.25 mmol:1.25 mmol, 12.5 equiv.) and DIEA (2.5 mmol, 25 equiv.) in NMP. Reactive side chains were protected as follows: Arg, pentamethyldihydrobenzofuran (Pbf) sulfonylamide; Asn and Gln, trityl (Trt) amide; Ser, Thr and Tyr, tert-butyl (tBu) ether; Asp and Glu, O-tert-butyl (OtBu) ester, and Lys and Trp, tert-butyloxycarbonyl (Boc) carbamate. Peptides were deprotected and cleaved from the resin by TFA as previously described [11,35]. Crude peptides were purified by reversed-phase HPLC (RP-HPLC) on a Vydac 218TP1022 C18 column (2.2 cm × 25 cm; Grace Discovery Sciences Alltech, Templemars, France) using a linear gradient (10–50% over 50 min) of acetonitrile/TFA (99.9:0.1, v/v) at a flow rate of 10 ml/min. Peptides were analyzed by RP-HPLC on a Vydac 218TP54 C18 column (0.46 cm × 25 cm; Grace Discovery Sciences Alltech) using a linear gradient (10–60% over 25 min) of acetonitrile/TFA at a flow rate of 1 ml/min. The purity of all peptides was higher than 99.7%. All peptides were characterized by MALDI-TOF mass spectrometry on a Voyager DE-PRO (Applera, France) in the reflector mode with α-cyano-4-hydroxycinnamic acid as a matrix.

2.3. Behavioral studies

2.3.1. Animals

Experiments were carried out on male Swiss albino mice (IFFA-CREDO/Charles River, Saint-Germain sur l'Arbresle, France) weighting 20–25 g. The animals were housed in Makrolon cages (L = 40 cm, l = 25 cm, h = 18 cm; 20 mice per cage) with free access to a standard semisynthetic laboratory diet (Ref AO4, UAR, Villemoisson-sur-Orge, France) and tap water. The animals were kept in a ventilated room at a temperature of 22 ± 1 °C, under a 12 h light/dark cycle (light on between 7:00 a.m. and 7:00 p.m.).

All the experiments were carried out between 9:00 a.m. and 4:00 p.m. in testing rooms adjacent to the animal rooms. Animal manipulations were performed in compliance with the European Communities Council Directive of the 24 November 1986 (86/609/EEC), were approved by the Regional Ethical Committee for Animal Experimentation (authorization numbers: N/01-05-04-20, N/10-04-04-12 and N/12-04-04-14) and were conducted by authorized