Summary of Ph.D. thesis

Structure-activity studies of novel, conformationally restricted delta opioid-receptor selective tetrapeptides

Ioja Enikő

Supervisiors
Prof. Dr. Anna Borsodi
Dr. Benyhe Sándor

Institute of Biochemistry
Biological Research Centre
Hungarian Academy of Sciences

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INTRODUCTION

Opiates are natural alkaloids derived from the poppy plant - *Papaver somniferum* – and considered the best analgesic compounds. Endogenous compounds with opiate-like biological activities are also existing in the nervous systems and other organs, termed opioid peptides. The occurrence of three major opioid receptor types (μ, δ, κ) interacting with the natural and synthetic opioid ligands has been also proved. Clinically used opioid analgesics act predominantly on μ-opioid receptors. It has been demonstrated that drugs acting on δ-opioid receptors also exhibit strong analgesic activities, but produce fewer side effects. Therefore a number of ongoing efforts are aimed to develop δ agonist ligands as centrally acting analgesics. An important discovery was the demonstration that δ-antagonist ligands can reduce the development of analgesic tolerance and severity of the precipitated withdrawal syndrome in mice. This observation led to the suggestion that opioid compounds with complex μ-agonist/δ-antagonist profile might be analgesics with a low proclivity to produce tolerance and dependence. The discovery of the prototype δ opioid antagonists TIPP (H-Tyr-Tic-Phe-Phe-OH) and TIP (H-Tyr-Tic-Phe-OH) in 1992 was followed by extensive structure-activity relationship studies, leading to the development of novel analogues with applications in opioid research and pharmacology. The first known peptide from the TIPP peptide series with mixed μ-agonist/δ-antagonist characters was the C-terminally amidated tetrapeptide TIPP-NH_2 (H-Tyr-Tic-Phe-Phe-NH_2). Further structure-activity

studies revealed that the δ-antagonist vs μ-agonist behavior of TIP(P) derived compounds depended on very subtle structural differences in diverse locations of the molecule and suggested a δ-receptor model involving a number of different inactive receptor conformations. Various substitutions were carried out in different sequences in these peptides such as the replacement of Phe^3 with β-methylphenyalanine resulting in an extraordinary δ-selectivity. The effects of methylation of the β-carbon of the third side chain on the biological properties of a peptide depends on the side-chain configurations of the stereoisomers. More interestingly, saturation of the Phe^3 aromatic ring in TIPP, as achieved through substitution of cyclohexylalanine (Cha), led to a compound (H-Tyr-Tic-Cha-Phe-OH) [TICP] with substantially increased δ-antagonist potency and higher δ-selectivity comparing with the parent TIPP peptide.

In the present thesis biochemical characterization of a series of novel TIPP related, structurally modified tetrapeptide ligands is described, reporting the results of extensive *in vitro* studies. Altogether 10 novel TIPP related compounds were investigated, with different substitutions such as N-terminal replacement of tyrosine by Dmt (2',6'-dimethyltyrosine) at the first position, and β-methyl-aminoacid substitution at the third position. Two of the novel tetrapeptides (DYTPP) were substituted with β-methylphenyalanine in place of Phe^3 beside the above mentioned one resulting in two diastereomeric compounds one of them the Dmt-Tic-(2S,3R)βMePhe-Phe being studied in radiolabelled form as well. The latter eight tetrapeptides (DYTCP stereomers – Dmt-Tic-βMeCha-Phe-Phe) carry β-

methylcyclohexyalanine substitution at the third position as well as four of the stereomers have amidated C-termini. Amidation of the C-terminus is expected to enhance μ-opioid receptor affinity of the ligands as it was demonstrated in the case of the DYTCP amidated compounds as well. The tritiated analogue was shown to label opioid δ-sites in rat brain and CHO cell line stably expressing δ-opioid receptors. The nonlabeled tetrapeptides were tested with highly selective radioligands [^{3}H]DAMGO (μ) and [^{3}H]Ile^{5,6}-deltorphin-II (δ) in competition binding assays. The novel analogues presented very interesting and complex intrinsic efficacies in rat brain membranes as well as in different cellular systems. The results obtained with the radiolabeled [^{3}H]Dmt-Tic-(2*S*,3*R*)βMePhe-Phe compound fulfill the main criteria for a valuable radioligand. Regarding the nonlabeled stereomeric compounds they might serve as valuable pharmacological and therapeutic agents as well as probes for μ- and δ-receptor coupling.

AIMS OF THE STUDIES

Recently a new series of highly potent, δ-receptor-selective opioid antagonist have been developed based on N-terminal message domain of the endogenous enkephalins (Tyr^1-Gly^2-Gly^3-Phe^4-) and amphibian skin derived dermorphins and deltorphins (Tyr^1-D-Xaa^2-Phe^3-, where Xaa^2 is Met or Ala). These new antagonist contain at least three aromatic residues; one of them being 1, 2,3,4-tetrahydroisoquinoline-3-carboxilic acid (Tic). The combination of

2',6'-dimethyltyrosine (Dmt) and Tic produced peptides with enhanced δ-receptor potency and selectivity. Further modifications of these peptides resulted in more complex compounds which beside the high δ-antagonist profiles displayed μ-agonist/δ-antagonist characters as well. The aim of the present study was to test biochemically and functionally several newly developed TIPP derived peptide analogues acting mainly through δ-opioid receptors as well as to study their structure-activity relationship. In the first part the structurally novel [^{3}H]Dmt-Tic-(2*S*,3*R*)βMePhe-Phe diastereomeric TIPP derived radiolabeled analogue was biochemically analysed. The main goals were:

- To have a novel opioid radioligand with improved activity and specificity.
- To measure its opioid activity in kinetic, equilibrium and competition binding studies.
- To compare its opioid binding properties with those of other well-known compounds labeling opioid receptors.

Furthermore the novel TIPP derived nonlabeled diastereomeric tetrapeptide ligands were biochemically and functionally analysed:

- To investigate their binding properties to the native receptors.
- To examine the post-binding effects (G-protein activation) in the functional biochemical [^{35}S]GTPγS binding assay.
- To elucidate their intrinsic characteristics in native as well as in different cellular systems.

EXPERIMENTAL PROCEDURE

Structural modifications on the TIPP related peptides as well as the radiolabeling were performed in the Biological Research Centre Opioid Receptor Research group, Isotope laboratory, led by Dr. Tóth Géza as well as in the Department of Organic Chemistry, Free University of Brussels, Belgium. For the biochemical and functional characterization of the novel tetrapeptides, a series of *in vitro* experiments were performed. The assays were carried out in Wistar rat brain membranes and Chinese hamster ovary cell lines stably expressing mouse δ- (δ_mCHO) or human μ-opioid (μ_hCHO) receptors. For the in vitro characterization and the structure-activity studies:

- Radioligand binding assays (Stauration, Displacement binding)
- [^{35}S]GTPγS binding assays were performed.

SUMMARY OF RESULTS

Since it was demonstrated that the δ-receptor sites are involved in the process or phenomenon of morphine tolerance and dependence the focus is on the development of potent antagonist ligands with high selectivity for the δ-receptors. Recently a new series of highly potent, δ-receptor selective opioid antagonist tetrapeptide ligands have been reported. The prototype antagonist ligand from these new peptides was TIPP (*Tyr-Tic-Phe-Phe*) which was followed by extensive structure-activity relationship studies. The present work focused on

the biochemical and functional analysis of a series of newly developed peptides from the TIPP series. The novel analogues carry different structural and conformational changes as substitution of the first Tyr by 2',6'-dymethyltyrosine (Dmt), the unnatural 1, 2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic[2]), β-methyl-amino acid substitution at the third place as well C-terminal modification. In this work we report the combination of Dmt[1] and β-Me-amino acid[3] substitution on the TIPP template structure and analyse the receptor binding and G-protein activating properties of the novel peptide analogues. The main findings are the following:

1. Side chain methylation of Phe by βMePhe[3] yielded diastereomeric pairs of analogues wherein Dmt-Tic-(2*S*,3*R*)βMePhe-Phe analogue was a potent δ-selective antagonist compound and the Dmt-Tic-(2*R*,3*S*)βMePhe-Phe had higher μ-receptor affinities and less δ-selectivity.

2. Specific binding of [^{3}H]Dmt-Tic-(2*S*,3*R*)βMePhe-Phe to rat brain membranes and to membrane fractions of CHO cell lines stably expressing δ-opioid receptors was of high affinity, saturable and stereoselective.

3. Kinetic experiments showed that the ligand-receptor association occurred rapidly according to pseudo-first order kinetics. Equilibrium saturation experiments revealed that a single class of nanomolar affinity opioid-binding sites was labeled by this radioligand.

4. Heterologous competition assays with different opioid compounds revealed that the rank order of potency is: δ > μ> κ in displacing [^{3}H]Dmt-Tic-(2*S*,3*R*)βMePhe-Phe specific binding.

5. Side chain methyl substitution by βMeCha^3 resulted in four diastereomeric DYTCP compounds displaying good δ-receptor affinities and different selectivities in function of their diastereomerism.

6. C-terminal amidation of the DYTCP stereoisomers slightly increased their μ-receptor affinity and μ-agonist properties.

7. Combination of Dmt^1 and βMePhe^3 replacements in the DYTPP ligands resulted in potent and more complex analogues displaying simultaneously δ-antagonist/agonist as well as μ-agonist/antagonist profiles in cell membrane preparations.

8. The βMeCha^3 substitution together with the Dmt^1 at the first position and C-terminal carboxamide group resulted in DYTCP stereoisomeric ligands with weak inverse agonist activities in rat brain and cell membranes as well as mixed μ-agonist/δ-antagonist properties in cell membrane preparations *in vitro*.

In conclusion the novel tetrapeptide analogues carrying different structural modification by their complex intrinsic properties may serve as valuable compounds in the biochemical and pharmacological research of the opioid system. Furthermore by their increased hydrophobicity imposed by dimethylation of Tyr as well as lipid solubility and low molecular weight might fulfill the suggested criteria required for passage through blood-brain barrier thus they might further offer new compounds for clinical studies. Moreover,

analogues from the TIPP family by their structural resemblance with endomorphins (Endomorpin 1: Tyr-Pro-Trp-Phe-NH_2 and endomorphin 2: Tyr-Pro-Phe-Phe-NH_2) might also r epresent a n interesting series for molecular modeling and searching for pharmacophore elements 'since a single small structural modification can d rastically c hange the l igands' intrinsic properties a nd characteristics.

LIST OF PUBLICATIONS

1. Enikő Ioja, Géza Tóth, Sándor Benyhe, Dirk Tourwé, Antal Péter, Csaba T ömböly, Anna B orsodi. Opioid r eceptor bi nding characteristics and structure-activity studies of novel tetrapeptides in the T IPP (Tyr-Tic-Phe-Phe) s eries. ***Neurosignals*** 2005; 1 4(6):317-328

2. Géza T óth, **Enikő Ioja**, Csaba T ömböly, S teven B allet, D irk Tourwé, Antal P éter, Tamás M artinek, N ga N . C hung, P eter W. Schiller, S ándor B enyhe, Anna B orsodi. β-Methyl s ubstitution o f cyclohexylalanine i n D mt-Tic-Cha-Phe pe ptides r esults i n hi ghly potent δ opioid antagonists. ***J. Med. Chem.*** 2007; 50(2):328- 333

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