Summary of Ph.D. thesis

Biological activity of structurally modified opioid receptor ligands

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Introduction

Opioid receptors are the members of G-protein coupled receptor superfamily. The binding of opioid agonists to the receptor triggers a chain of signaling events, which are involved in the cellular mechanisms of pain control and drug addiction. The existence of three major types of opioid receptors, designated as mu, delta and kappa, is generally accepted. The synthesis of new, selective ligands with high affinity for a specific type of receptors is one of the important directions in the field of opioid research. New compounds can be employed as both pharmacological tools and potential therapeutic agents.

This thesis describes the characterization of several newly developed ligands acting preferably on mu and delta opioid receptors based on biochemical (radioligand binding) and functional (guanosine-5'-[γ-^{35}S]-triphosphate ([^{35}S]GTPγS) binding) assays. Rat brain membrane preparations were used in these *in vitro* studies.

Ligands acting through delta opioid receptors play an important role in antinociception, immunoregulation, and can be useful for treatment of alcohol and drug addicts. Non-peptide compounds are of a big importance because of their ability to penetrate blood brain barrier and relative stability against enzymatic degradation. There is a big emphasis on the development of new selective antagonists, which, except for medicinal purposes, are used to evaluate the selectivity of new compounds and to study the interaction of endogenous ligands with different opioid receptors. In this study new non-peptide compounds based on the structure of naltrindole and naltriben, well known delta opioid antagonists, were investigated. According to the

structural modifications it was expected that new compounds would demonstrate improved selectivity and/or affinity towards delta opioid receptor.

Until present days the majority of drugs clinically used for the pain control are mu opioid receptor selective ligands. Etorphine is a highly potent synthetic narcotic compound, which exerts its action mainly through mu opioid receptors. It is known to cause strong analgesia, catatonia and blockade of conditioned reflexes in laboratory animals. Etorphine is widely used for the immobilization of different types of animals in veterinarian and nature preservation practice. Since C18 β-substituted structural analogues of etorphine compounds have not been studied until now, it is of considerable interest to investigate their features. Therefore a number of new β-substituted etorphine ligands were synthesized and their binding and functional characteristics were examined in this study.

Endomorphin 1 and 2 (Tyr-Pro-Trp-Phe-NH_2 and Tyr-Pro-Phe-Phe-NH_2, respectively), two peptides which demonstrated high affinity and selectivity for mu opioid receptor, were isolated from bovine brain in 1997. Later endomorphins were found in human brain and immune tissues. Endomorphins, like other mu opioid receptor agonists, produce strong analgesic effect and were shown to take part in regulation of cardiovascular activity. Endomorphins were shown to act through classical mu-opioid receptor pathway and activate G-proteins and inhibit adenylyl cyclase activity. In the present study we investigated how the enzymatic degradation would affect the binding and functional properties of endomorphins and the role of C-terminal of these peptides for efficient binding to opioid receptors.

Aim of the studies

To investigate structure-activity relationships of newly developed analogues of well known non-peptide delta selective antagonists naltrindole and naltriben; to improve the selectivity and/or affinity of the resulted indolo- and benzofuromorphinans with focus on the 5- and 14-positions.

To investigate how structural modifications introduced to highly potent synthetic opioid compound etorphine will affect biochemical and functional properties of the ligands. To examine the effects of C18 β-substitution.

To assess the effects of possible enzymatic degradation of endomorphin 1 and the influence of C-terminal structural modifications of endomorphin 2 on receptor binding and the consequent activation of G proteins.

Methods

Competition binding experiments

Binding experiments were carried out in 50 mM TRIS-HCl buffer (pH 7.4) in a final volume of 1 ml containing 0.3-0.4 mg protein. In experiments with [^{3}H]EKC 100 nM DAMGO and [D-Ala^2,Leu^5]enkephalin were added to the reaction mixture to block mu and delta binding sites. Incubations were started by addition of membrane suspension and terminated by rapid filtration using Brandel Cell Harvester through Whatman GF/B or GF/C

glass fibre filters and washed three times with 5 ml of ice-cold TRIS-HCl (50 mM, pH 7.4) buffer. The filters were dried at 37°C and the bound radioactivity determined in a toluene based scintillation cocktail using Wallac 1409 scintillation counter. All experiments were carried out in duplicates and repeated at least three times.

[^{35}S]GTPγS binding

Tubes containing 10 μg protein, 30 μM GDP, opioid ligand and 0.05 nM [^{35}S]GTPγS, all in 50 mM Tris-HCl buffer containing 1 mM EGTA, 100 mM NaCl and 3 mM $MgCl_2$ in a final volume of 1 ml were incubated for 1 h, at 30°C. Nonstimulated activity was measured in the absence of the tested compound, nonspecific binding was measured in the presence of 100 μM unlabelled GTPγS. The reaction was started by addition of [^{35}S]GTPγS, and terminated by filtrating the samples through Whatman GF/B glass fiber filters. Filters were washed three times with ice-cold 50 mM Tris-HCl buffer (pH 7.4) using Brandell Cell Harvester, then dried, and radioactivity was measured in a Wallac 1409 scintillation counter (Turku, Finland) using a toluene based scintillation cocktail. Stimulation is given as percent of the specific binding. Data were calculated from at least three independent experiments performed in triplicates.

Results and conclusions

All new indolo- and benzofuromorphinan compounds displayed high affinity for delta opioid binding sites. The compound with a methyl group at the 17-N position was found to be an agonist, thus confirming the

importance of the substituent at the morphinan nitrogen for agonist/antagonist features. All other compounds were antagonists. The presence of a methyl group in position 5 induced no change in delta affinity, but decreased the mu and kappa affinities. An ethoxy group at position 14 conferred a very high affinity and also high selectivity to delta opioid receptors. Chain prolongation of the 14-alkoxy group resulted in compounds with reduced delta affinity and selectivity. The results demonstrate that 5- and 14-positions of indolo- and benzofuromorphinans represent critical sites that can be successfully used to develop new compounds with increased affinity and selectivity for delta binding sites.

All new etorphine derivatives showed high affinity for mu opioid receptors. β-Substituted etorphines had slightly lower affinities compared to the parent α-etorphines. Methylether derivatives were consistently weaker than the corresponding phenolic compounds. Dihydroetorphine and β-dihydroetorphine having partially saturated ring structure showed as good potency in the binding assays as did etorphine and β-etorphine with C7-C8 double bonds. It was also found that C3 phenolic group is far more favorable for G-proteins activation. The results suggest that neither the configuration of C18 nor the saturation of the C7-C8 double bond play a critical role in the biological activity of etorphines.

Both N- and C-terminal truncated endomorphin 1 derived peptides demonstrated considerably lower opioid receptor binding potency compared to the parent endomorphin 1. None of the truncated peptides had an effect on

GTP binding. Obtained results suggest that degradation destroys the biological activity of endomorphin 1.

Peptides obtained as a result of the alteration of the C-terminal carboxamide group of endomorphin 2 generally demonstrated decreased affinity towards mu opioid receptor. The exception was E2-ol, where the amide group was replaced with an alcohol that resulted in increased affinity for mu binding sites. All derivatives had lower functional activity than parent endomorphin 2. It has been shown that the distance between the C-terminal aromatic ring and the peptide backbone has strong effect on the receptor binding and the functional activity of the peptides. Although the C-terminal carboxamide group can be eliminated from the molecule without serious loss of binding activity, for efficient receptor stimulation and naloxone antagonism at least one polar group is necessary at the C-terminus. The data presented demonstrate that the C-terminal amide group has an essential role in the regulation of the binding and the agonist/antagonist properties of E2.

Publications

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