Biological activity of structurally modified opioid receptor ligands

Ph.D. thesis

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TABLE OF CONTENTS

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ABBREVIATIONS

ACTH	adrenocorticotrop hormone
ADP	adenosine-5'-diphosphate
BSA	bovine serum albumin
cAMP	adenosine-3,5'-cyclic monophosphate
CNS	central nervous system
DAMGO	[D-Ala^2(Me)Phe^{4}Gly^{5}ol]enkephalin
E1, E1-NH_2	endomorphin 1
E2	endomorphin 2
EC_{50}	effective concentration 50%; the concentration of a drug that gives 50% of maximal response
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N',-tetraacetic acid
EKC	ethylketocyclazocine
Et	ethyl
GDP	guanosine-5'-diphosphate
GPCR	G protein-coupled receptor
G-protein	guanine nucleotide binding protein
GTP	guanosine-5'-triphosphate
GTPγS	guanosine-5'-O-(γ-thio)triphosphate
IC_{50}	inhibitory concentration 50%; the concentration of a drug that inhibits 50% of the specific binding of a competing ligand
K_i	equilibrium inhibition constant
Me	methyl
MSH	melanocyte stimulating hormone
NTB	naltriben
NTI	naltrindole
Nx	naloxone
PLC	phospholipase C
POMC	proopiomelanocortin
PTX	pertussis toxin
SEM	standard error of the mean
Tris	tris-(hydroxymethyl)-aminomethane

Standard one- and three-letter codes for amino acids were used

SUMMARY

Opioid receptors, which belong to G protein-coupled receptors, are famous for the important role they play in analgesia, as well as in drug addiction. Further research is needed to improve our understanding of the mechanisms, underlying these processes. In the present study we investigated how structural modifications of the known delta (naltrindole and naltriben) and mu (etorphine, endomorphin 1 and 2) specific ligands influence their binding characteristics and biological activity. *In vitro* radioligand binding and functional ([^{35}S]GTPγS binding) assays were performed in the rat brain membranes.

Non-peptide antagonists are of a big importance for scientific and clinical purposes. The development of stable highly delta-selective non-peptide ligands is required. To approach this goal the structure of well known delta opioid antagonists naltrindole and naltriben was modified and the structure-activity relationships of newly developed indolo- and benzofuromorphinans was investigated. The results indicate that the presence of a 5-Me group induced no change in delta affinity, but decreased the mu and kappa affinities, thus increasing mu/delta and kappa/delta selectivity ratios. The substitution at position 14 proved to be a very important determinant in increasing delta selectivity. An ethoxy group in this position confers a very high affinity and also high selectivity to delta opioid receptors. The importance of the morphinan nitrogen substituent for agonist/antagonist character of the compounds was demonstrated in both radioligand binding and functional assays. Obtained results demonstrate that the positions 5 and 14 of indolo- and benzofuromorphinans represent critical sites that could be a trigger to develop new compounds with increased delta affinity and/or selectivity.

The highly potent synthetic narcotic compound etorphine exerts its effects largely through mu opioid receptor. A number of new structural analogues to etorphine, including C18-β-structures, 3-O-methylether derivatives and saturated C7-C8 dihydro-compounds were examined. The newly synthesized β-etorphine analogues have high affinities in [^{3}H]naloxone binding assays and they potently stimulate [^{35}S]GTPγS binding. Both radioligand binding studies and receptor-mediated activation of the G-proteins strongly suggest the agonist nature of all the compounds tested. 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.

The recently isolated endogenous peptides endomorphin 1 and 2 have high affinity for the mu opioid receptor and play an important role in analgesia. Several of its degradation products have been isolated from the central nervous system. Degradation products present structural similarities and may influence the receptor binding properties and biological activity of the parent compound. Therefore, we investigated how degradation of endomorphin 1 might influence ligand binding to the mu opioid receptor and the consequent activation of G proteins. Both N- and C-terminal truncation of endomorphin 1 resulted in peptides presenting considerably lower opioid receptor binding potency. None of these peptides had an effect on GTP binding, suggesting that degradation destroys the biological activity of endomorphin 1. C-terminal modifications were introduced to endomorphin 2 to improve its binding properties and biological activity and to further our understanding of how structural modifications might influence the binding and functional properties. The alteration of the C-terminal carboxamide group mostly decreased the affinity of the peptides to the mu opioid receptor, except for endomorphin 2-ol, where the amide group was replaced with an alcohol. All derivatives had lower functional activity than parent endomorphin 2. The data presented demonstrate that the C-terminal amide group has essential role in the regulation of the binding and the agonist/antagonist properties of E2.

1. INTRODUCTION

1.1. The history of opium and opioids

The ability of poppy plant (*Papaverum somniferum*) to produce mood-altering and sleep-inducing effects has been known for thousands of years. Poppy is mentioned in Sumerian records (5000 to 4000 BC) and Assyrian tablets refer to medicinal properties of opium (name coming from "*opos*", Greek word for juice), substance obtained from the unripe seed pods of the poppy plant. There is an opinion that mysterious drug "nepenthe" ("against sorrow") which, according to Homer, "drowning cares and angers, did decline / All thought of ill. Who drunk her cup could shed / All that day not a tear..." (Odyssey IV, 295-297, translated by George Chapman) was also nothing else but opium. Famous physician, writer, and philosopher Galen (AD 129 – ca. 216) was of very high opinion of the virtues of opium, and his writings greatly influenced European medical theory and practice. Later Paracelsus (1493–1541) introduced the alcoholic tincture of opium called *laudanum* as an analgesic and sedative. *Paregoric*, another opium-based medicine widely used for the treating of diarrhea, was made by combining camphor with tincture of opium. Since there were few alternative therapeutics or painkillers until the 19th century, opium was somewhat of a medical panacea. Opium and opium-based products were easily available and widely prescribed by physicians.

The habit of opium smoking began after importing into Europe the practice of North American Indians to smoke tobacco in pipes. Gradually opium smoking became a preferred method of taking the drug.

Morphine (named after the Greek god of dreams Morpheus), the first of 20 or more alkaloids found in opium, was isolated in the very beginning of the 19th century by the young German pharmacist Serturner. The isolation of other alkaloids, such as codeine and papaverine, followed soon. These pure products fast earned popularity in the medical world. In 1853 a French physician Charles Pravaz invented the hypodermic syringe, and it made possible the administration of morphine by injection. This way the effect of the drug is much stronger than when the same amount is taken orally.

The fact that the use of opium leads to addiction is known since antiquity. However, it seems that this did not create serious social problems until comparatively recent times. The situation changed with the use of hypodermic needle for intravenous injections of opioids. During the Civil War in the U.S.A. about 400,000 soldiers treated with opiates became addicted. With the synthesis of more powerful drugs the problem further escalated. Thus, the search for better cough, chest and lung medicine led the Bayer Company to the synthesis of heroin (the name derived from the adjective "heroisch") in 1898. There was considerable interest in the highly effective new drug, which was initially declared to be non-addictive. Unfortunately, heroin, though being a more potent and faster acting painkiller than morphine, soon was found to be even more addictive.

Opioid addiction soon became a serious political concern, leading to the Opium Convention that was signed in the Hague in 1912 to control opium trade. Its conditions were added to the Treaty of Versailles after the end of World War I. In the U.S. all domestic manufacture of heroin was banned in 1924. In Europe the League of Nations basically drove heroin manufacture underground by the early 1930s.

Despite all efforts the situation with opiate and other narcotic abuse remains a serious problem worldwide. According to the U.S. National Household Survey on Drug Abuse, in 1999 an estimated 208,000 Americans were current users of heroin, more than tripling the number since 1993, with rapidly dropping the average age of heroin users. Heroin-related deaths are rising as a result of the increasing purity and decreasing price of the drug. UN World Drug Report 2000 says that "the UN estimates that some 180 million people worldwide – 4.2 per cent of people aged 15 years and above – were consuming drugs in the late 90s; this figure includes 13 million people abusing opiates, 9 million of whom were addicted to heroin".

This statistics clearly shows the necessity of scientific search for new alternative drugs, which ideally would be able to produce analgesia without undesirable side effects and help in curing the destructive addiction.

1.2. Opioid receptors

1.2.1. Opioid receptors: discovery, heterogeneity

In the end of the 19th century Fisher proposed the lock-and-key model for the enzyme-glycozide system. This idea was developed further and applied for receptors as well. The term "receptor" was introduced by P. Ehrlich. In general, receptors are protein molecules, components of a cell or organism, which interact with a drug and initiate a stream of biochemical reactions. Receptor-mediated drug effects involve two processes: binding (i.e. ligand-receptor complex formation) and receptor activation, which mediates the effect. All ligands, which upon binding to a receptor produce certain response, are called agonists; compounds, which bind to the receptor but produce no effect, are called antagonists. Term "opiate" is usually used for the ligand of non-peptide nature, and "opioid" is a broader term describing both peptide and non-peptide compounds.

Different pharmacological evidence led to the idea of the existence of specific receptors for opioid ligands in central nervous system (CNS). First demonstration of these receptors was done by three independent research groups in 1973 (Simon 1973, Terenius 1973, Pert 1973). In 1976 experiments on spinalized dogs performed by Martin and coworkers clearly showed that opioid receptors are not a homogeneous group, as it was believed before, but consist of several different types. According to the drugs used in the experiments, three types of receptors discovered were named mu (μ, for morphine), kappa (κ, for ketocyclazocine) and sigma (σ, for SKF 10,047). Next year Lord et al (1977) described one more opioid receptor type found in the mouse *vas deferens* preparation, and this type was named delta (δ) receptor. Later sigma receptor was found to be non opioid, and by now only mu, delta and kappa receptor types are widely accepted, although several other receptor types were proposed as opioid receptors (epsilon (ε, Wuster et al., 1979), lambda (λ, Grevel et al., 1985) and zeta (ζ, Zagon et al., 1991)). For each type of opioid receptors several subtypes are described (Wolozin and Pasternak, 1981; Jiang et al., 1991; Sofuoglu et al., 1993; Wollemann et al., 1993). For the nomenclature of opioid receptor see Table 1.1.

Opioid receptors are present in the central nervous system and peripheral tissues, though the exact patterns of their distribution, proportion of different types of receptors

and their quantity varies between different species and anatomical regions (Mansour et al., 1988; Lutz and Pfister, 1992).

Table 1.1. Nomenclature of opioid receptors

Opioid Receptors		
Pharmacology Nomenclature	IUPHAR Recommendation	Molecular Biology Nomenclature
delta	OP1	DOR, OPRD
kappa	OP2	KOR, OPRK
mu	OP3	MOR, OPRM

Opioid receptors are involved in different physiological processes (Table 1.2.). Analgesia is the most important pharmacological effect of opioid receptor activation. Different autonomic responses such as respiratory depression, nausea, bradychardia, thermal regulation, antidiuretic responses, etc., are also mediated through opioid receptors.

Table 1.2. Main effects mediated by opioid receptors

Effect	Receptors		
	mu	delta	kappa
Analgesia	+++	++	++
Euphoria	+++	+	±
Dysphoria	±	±	+++
Respiratory depression	+++	±	+
Constipation	+++	±	±

+++ = pronounced effect, ++ = significant effect, + = some effect, ± = little or no effect

1.2.2. Structure of opioid receptors

The first cloning of opioid receptors was performed in 1992 (Kieffer, 1992; Evans, 1992). Before this major breakthrough in opioid research area few facts were

known about the exact nature of opioid receptors. Opioid receptors from different sources were solubilized and found to be glycoproteins (Ruegg et al., 1980; Simonds, 1980; Howell, 1982; Gioannini, 1982), but whether different types of receptors were different proteins or not, was still unknown.

Soon after successful cloning of delta receptors, the cloning of kappa and mu opioid receptors was reported (Yasuda et al., 1993; Chen et al., 1993a,b; Wang et al., 1993; etc.). Sequence analysis of the cloned receptors proved that they belong to the superfamily of G protein-coupled receptors (GPCR). Thus, opioid receptors share GPCR's classical structure: seven transmembrane spanning helices connected by intra- and extracellular loops (Fig. 1.1.). Opioid receptors were shown to have an extracellular N-terminal with multiple glycosilation sites, third intracellular loop with multiple amphiphatic α-helices and forth intracellular loop with putative palmitoylation sites (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Fukuda et al., 1993).

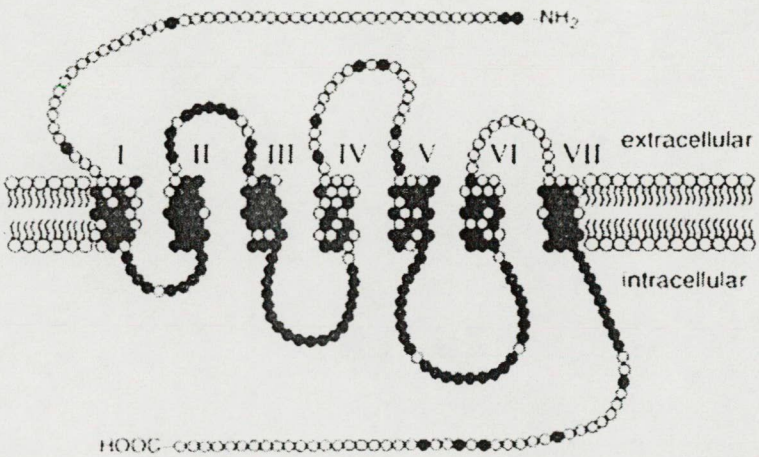


Fig. 1.1. Schematic model of an opioid receptor. Each circle represents an identified amino acid. Amino acid residues that are identical or similar among the receptors represented by closed circles, those that are not similar by open circles.

The amino acid sequences of mu, delta and kappa receptors are approximately 60% similar to each other, with the highest homology found in transmembrane domains, intracellular loops and a portion of the C-terminal tail adjacent to seventh transmembrane

domain. The third intracellular loop is implicated in binding G proteins and has consensus sequences for phosphorylation, which might be involved in regulatory processes as G protein docking. High homology of this region suggests that various opioid receptors may interact with similar G-protein complexes. The greatest divergences in amino acid sequence occur mostly in the N-terminus, second and third extracellular loops and the C-terminus.

1.2.3. Opioid receptors and signal transduction

G-proteins are trimeric proteins that mediate signal transduction through the GTPase cycle. They consist of α, β and γ subunits. The interaction of a ligand with the receptor results in conformational changes of the latter. The changes trigger GDP dissociation from the α subunit of the G protein, bound to the receptor. Binding of GTP to the empty binding pocket of α subunit results in dissociation of the α subunit from G protein and βγ complex, what in turn initiates a number of signal transduction pathways.

Opioid receptors belong to "G_i / G_o–coupled" receptors because opioid signals are efficiently blocked by pertussis toxin (PTX), a bacterial toxin that ADP-ribosylates and inactivates the α subunits of G_i / G_o proteins. Opioid receptors are known to be involved in inhibition of adenylyl cyclase (Sharma et al., 1977), regulation of Ca^{2+} (Hescheler et al., 1987; Surprenant et al., 1990; Jin et al., 1992) and K^+ channels (North et al., 1987), and phospholipase C activity (Smart et al., 1994). Recently, the opioid receptors were shown to regulate the mitogen-activated protein (MAP) kinase cascade (Li and Chang, 1996; Fukuda et al., 1996; Burt et al., 1996).

Since PTX was shown originally to reverse the inhibition of adenylyl cyclase in neuroblastoma×glioma NG 108-15 cells (Hsia et al., 1984) and rat brain receptors were solubilized as a complex with PTX-sensitive G-proteins (Wong et al., 1989), it was believed that opioid receptors act through G_i proteins. Later it was shown that mu, delta and kappa receptors could activate $G_{i/o}$ proteins with equal potency (Raerig et al, 1992; Prether et al., 1995; Chakrabarti et al., 1995).

Another member of G_i subfamily, PTX-insensitive G-protein, G_z, also is able to inhibit cAMP accumulation upon receptor activation (Wong et al., 1992). It is possible that G_z may play some role in opioid signal transduction (Law et al., 2000).

Interestingly, there are reports suggesting that the complexity of the adenylyl cyclase system allows different routes for opioids not only to inhibit, but also to stimulate cAMP production (Sarne et al., 1998). Thus, Gβγ complex was demonstrated to stimulate adenylyl cyclase of type 2, 4 and 7 (Federman et al., 1992; Tsu et al., 1995). Since adenylyl cyclase type 2 and 4 are expressed in the brain, it is quite possible that some of the central actions of the opioids are mediated by these enzymes. So, the regulation of intracellular cAMP by opioids seems to be rather complicated (Law et al., 2000).

Another mechanism of opioid receptor action is influencing ion channels. All three types of opioid receptors are able to inhibit different types of Ca^{2+} channels (Rhin et al., 1994; Connor et al., 1999). Cloned rat mu opioid receptor is functionally coupled to N-type Ca^{2+} channels. (Morikawa, 1995). Cloned mu and delta opioid receptors expressed in GH3 pituitary cells inhibited voltage-activated L-type Ca^{2+} channels (Piros et. al., 1996). Kappa opioid receptors also are able to modulate Ca^{2+} channels activity (Kaneko et al., 1994). Presently it is realized that Gβγ subunits inhibit Ca^{2+} channels rather than $G\alpha_o$ subunit (Ikeda, 1996; Herlitze et al., 1996).

Electrophysiological studies demonstrated that opioid receptors could modulate K^+ channels activity (North et al., 1987; Grudt and Williams, 1993; Shultz, 1998). There are data indicating that Gβγ subunits activate K^+ channels, and that Gβ subunit can interact with K_G channel subunits. Therefore it is possible that the association of different types of opioid receptors with distinct G-proteins (and, therefore, with different Gβ subunits) can be the reason for the different abilities of opioid receptors to activate K_G channels.

Phospholipase C (PLC) is another effector molecule for opioid receptors. Opioid stimulation of PLC was shown in SH-SY5Y human neuroblastoma cells (Smart et al., 1994). Mechanism of PLC activation may involve Gβγ subunits (Chan et al., 1995) or activation of L-type Ca^{2+} channels (Smart et al., 1995).

Recently opioid receptors were demonstrated to stimulate MAP-kinase cascades. All types of opioid receptors stimulated extracellular-signal – regulated kinases (ERK) type 1 and 2 (Li and Chang 1996; Burt et al., 1996; Fukuda et al., 1996), and the activation was shown to be mediated by Gβγ subunits in a Ras-dependent manner.

1.3. Opioid receptor ligands

1.3.1. Endogenous opioid ligands

When the opioid receptors were discovered the endogenous ligands acting upon these receptors were unknown. After opioid receptors were described in 1973 (Simon, 1973; Terenius, 1973; Pert, 1973), intensive search began for endogenous ligands that act on these receptors. The first two endogenous opioid pentapeptides were found in pig brain by Hughes et al. in 1975. These two peptides which differ only in the 5th amino acid (methionin or leucin) were named enkephalins (Table 1.3.). Discovery of other endogenous opioid peptides followed soon. In mammalian brain α, β and γ-endorphins were described in 1976 (Cox et al.), and dynorphins in 1979 (Goldstein et al.). All these peptides share a common characteristic sequence Tyr-Gly-Gly-Phe at their N-terminal. It was shown that these peptides come from three genetically different precursor peptides: proopiomelanocortin (POMC), proenkephalin and prodynorphin. POMC gives rise to endorphins, as well as non-opioid adrenocorticotrop hormone (ACTH) and melanocyte stimulating hormone (MSH) (Mains et al., 1977). Proenkephalin contains one copy of Leu-enkephalin, four copies of Met-enkephalin and two copies of extended Met-enkephalins. Prodynorphin gives rise to dynorphin A and B, and α-, β-neoendorphins.

All three precursors are nearly of the same length. The neuroactive peptides are mostly situated towards the carboxyl terminal end of the polyprotein precursor. It was shown that the neuroactive domains of the precursors are marked by pairs of basic amino acid residues that form potential cleavage sites for trypsin-like enzymes. It is found that the same precursor protein is present in several different tissues, but undergoes different post-translational processing. It seems likely that the differences in three precursors proteins arise from differences in the splicing of mRNA chains after the excision of introns (Smith, 1997).

Although endogenous peptides usually have some binding preferences to a certain receptor type, they are able to bind to all three types of opioid receptors. The enkephalins display modest selectivity for delta receptor type, but also cross-react with mu opioid receptors. Dynorphins predominantly bind to kappa receptors (Heyl and Schullery, 1997). Dermorphins and deltorphins, another groups of endogenous opioid peptides that were

found in the skin of amphibia *Phyllomedusa*, are mostly mu- and delta-selective ligands, respectively (Mignona et al., 1992; Erspamer et al., 1989). These two classes of amphibian peptides share a common N-terminal aminoacid sequence, Tyr-D-Xaa-Phe, where Xaa is either D-Ala, D-Met or D-Leu.

Endogenous opioid peptides are involved in a variety of physiological effects, and the most remarkable of them is analgesia. Other functions include respiratory depression, regulation of immune response, neurohormone release, mood changes, feeding behavior, and intestinal motility. Deltorphins induce behavioral stimulation (increase in locomotion and sniffing in rats) (Negri et al., 1991), display antidiarrheal properties and reduce body temperature.

In 1997, Zadina and colleagues isolated two new tetrapeptides from bovine brain, both of which had high affinity and selectivity for the mu receptor. These endogenous peptides were named endomorphin 1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin 2 (Tyr-Pro-Phe-Phe-NH_2). Later endomorphins were found in human brain and spinal cord (Hackler et al., 1997) and immune tissues (Jessop et al., 2000). Endomorphins, like other mu opioid receptor agonists, produce strong, though short lasting, analgesic effect (Stone et al., 1997; Soignier et al., 2000) and were shown to take part in regulation of cardiovascular activity (Champion et al., 1997; Czapla et al., 1998). Endomorphins were shown to act through classical mu-opioid receptor pathway and activate G-proteins and inhibit adenylyl cyclase activity (Narita et al., 1998; Monory et al., 2000). They were reported to be full agonist acting on mu opioid receptors (Gong, 1998), whereas other groups found them to be only partial agonists (Sim et al., 1998; Hosohata et al., 1998).

Though there are numerous reports of endomorphins exerting their effects through mu opioid receptors (for review see Horvath, 2000), there are some evidences, which do not support the idea of exclusive action of endomorphin 1 through mu opioid receptors, or suppose that it acts through some subtype of mu receptor. For example, endomorphin 1 induced antinociception was not reversed by mu-1 selective antagonist naloxonazine in mice (Sakurada et al., 1999). In experiments with diabetic mice mu opioid receptor antagonist beta-funaltrexamine and mu-1 subtype antagonist naloxonazine were ineffective in antagonizing endomorphin 1's antinociceptive effect (Kamei et al., 2000).

Table 1.3. Endogenous opioid peptides

Opioid peptide	Structure	Precursor
β -endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu	POMC
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu	Proenkephalin
Met-enkephalin	Tyr-Gly-Gly-Phe-Met	
Met-enkephalin-Arg^6-Phe^7	Tyr-Gly-Gly-Phe-Met-Arg-Phe	
dynorphin $A_{(1-8)}$	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile	Prodynorphin
dynorphin $A_{(1-13)}$	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys	
dynorphin $A_{(1-17)}$	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gly	
α-neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys	
β-neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro	
dermorphins	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	Frog skin peptides
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-OH	
	Tyr-D-Ala-Phe-Trp-Tyr-Pro-Lys-OH	
	Tyr-D-Ala-Phe-Trp-Asn-OH	
	Tyr-D-Ala-Phe-Trp-Tyr-Pro-Asn-OH	
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-Gly-Glu-Ala-OH	
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-Gly-Glu-Ala-Lys-Lys-Ile-OH	
deltorphins	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	
	Tyr-D-Ala-Phe-Asp-Val-Val-Gly- NH_2	
	Tyr-D-Ala-Phe-Glu-Val-Val-Gly- NH_2	
	Tyr-D-Leu-Phe-Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile-NH_2	
endomorphin 1	Tyr-Pro-Trp-Phe-NH_2	Unknown
endomorphin 2	Tyr-Pro-Phe-Phe-NH_2	

The experiments with endomorphin 1 stimulated viral replication in microglial cell culture also suggest that endomorphin 1 acts through "atypical" mu receptors (Peterson et al., 1999).

Experiments performed in our group suggest that endomorphin 1 is able to bind to both mu-opioid receptors and non-opioid naloxone-insensitive site, which exact nature and function are to be investigated (Borsodi et al., 2001).

The story of endogenous ligands for opioid receptors got an interesting twist after the discovery of morphine in the nervous system of both vertebrates and invertebrates (Sonetti et al., 1999; Bianchi et al., 1993). It is argued that endogenous morphin itself can play a role of signaling molecule acting through a mu_3 receptor subtype (Stefano et al., 2000).

1.3.2. Exogenous ligands for opioid receptors and approaches used for their development

The development of potent and stable agonists and antagonists with high specificity for each of the three major opioid receptor types is an important goal in opioid research. Both peptide and non-peptide compounds are widely employed for this purpose. There are several approaches employed to create new ligands.

The intensive investigation of compounds containing a functional reactive group, which can form irreversible bond with the receptor (affinity labels) in order to distinguish between different receptor types started soon after the discovery of multiple opioid receptors. For affinity labels that contain electrophilic group the covalent binding of the ligand to the receptor consists of two consecutive recognition steps. The first recognition step involves a reversible association of the ligand with the receptor; the second step, which leads to covalent bond formation, requires proper alignment of the electrophilic group with a receptor-based nucleophile (Portoghese, 1992). The second step should amplify the recognition of the affinity label when the electrophilic group is chemically selective and within covalent binding distance from of a compatible receptor-based nucleophile. This phenomenon is called recognition amplification. Based on this concept irreversible non-selective opioid agonist chloroxymorphamine (Caruso et al., 1979) and antagonist β-chlornaltrexamine (β-CNA) (Portoghese et al., 1978), as well as highly mu

selective antagonist β-funaltrexamine (β-FNA) (Portoghese et al., 1980) were synthesized. Both β-CNA and β-FNA are widely used as pharmacological tools.

Another approach to develop highly selective opioid ligands involves the linkage of two recognition units through a spacer (Erez et al., 1982), creating bivalent ligands. The rationale for this approach was based on the assumption that enhanced potency and selectivity may be conferred by bridging two neighboring recognition sites, which may be either on two neighboring receptors or two subsites on a single receptor (Portoghese, 1989). This approach lead to the synthesis of κ-opioid selective antagonists TENA, β-naltrexamin derivative (Portoghese and Takemori, 1985) and norbinaltorphamine (norBNI) (Portoghese et al., 1987).

Another concept proposed for synthesizing new opioid receptor ligands was based on Schwyzer's "message-address" concept (Schwyzer, 1977). According to this concept, originally created for understanding structure-activity relationships of ACTH and related hormones, peptide molecule contains a "message" sequence and "address" sequence of amino acid residues. The "message" part is responsible for signal transduction, and the "address" component provides additional binding affinity. It was proposed that N-terminal Tyr-Gly-Gly-Phe sequence of endogenous opioid peptides (endorphins, enkephalins, dynorphins) corresponds to the "message" and is responsible for opioid effect, and that C-terminal part of these peptides which carries different sequences functions as an "address" (Chavkin, Goldstein, 1981). The evaluation of this concept suggested that the Tyr^1 residue of the opioid peptides comprises the message component and that the sequence starting with Phe^4 serves as an address; in this context Gly^2-Gly^3 serves as a spacer (Portoghese, 1992). This is consistent with the structure of non-peptide opioid ligands such as morphine or oxymorphone, which contain only one aromatic ring presumably mimicking the Tyr^1. Based on the "message-address" concept δ-opioid receptor selective non-peptide antagonist naltrindole (NTI) was synthesized (Portoghese et al., 1988) using the naltrexone pharmacophore for the message moiety and joining it to the address element, which was believed to be the phenyl group through rigid pyrrole spacer. Replacement of the pyrrole spacer with furan lead to the creation of naltriben (NTB), also very potent δ-opioid receptor selective antagonist (Portoghese et al., 1991).

One more approach to synthesize selective opioid ligands is to modify or conformationally restrain already known compounds. For example, Hruby and coworkers reported the synthesis of pure opioid antagonists with high μ over δ selectivity by creating cyclic conformationally constricted peptides related to somatostatin (Pelton et al., 1985; Pelton et al., 1986). There were several attempts to develop κ-selective antagonists by structurally modifying endogenous opioid ligand dynorphin A (Lemair and Turcotte, 1986; Gairin et al., 1986).

1.4. Interactions between opioid receptors

Early biochemical and pharmacological studies suggested that distant mu and delta receptors coexisted in an opioid receptor complex (Rothman and Westfall, 1982). These observations led to the hypothesis that there are different subtypes of opioid delta receptors: associated with mu opioid receptors (termed complexed, or $delta_{cx}$) and not associated with mu receptors (non-complexed, $delta_{ncx}$) (Heyman et al., 1989; Rothman et al., 1989). Later other binding and behavioral studies have led to the proposal that $delta_{ncx}$ and $delta_{cx}$ receptors are synonymous with the $delta_1$ and $delta_2$ of another classification, however, other evidences accumulated suggesting the interaction between mu and delta opioid receptors. Behavioral studies suggest that mu and delta-opioid receptors can also interact to modulate the antinociceptive response. Thus, Vaught and Takemori (1979) showed that an intracerebroventricular injection of Leu-enkephalin potentiates the antinociceptive action of morphine. Later, it was shown that the antinociceptive action of morphine can be antagonized by Met-enkephalin (Lee et al., 1980; Vaught et al., 1982). Subantinociceptive doses of the delta opioid agonist DPDPE, being coadministered with intracerebroventricular mu-opioid agonist morphine, potentiate the antinociception produced by morphine. However, when other delta opioid agonists, such as Met-enkephalin, are coadministered, the antinociception produced by morphine is attenuated (Qi et al., 1990). All of these results indicate that antinociception can be modulated by an interaction between delta and mu receptors. O'Neil et al. (1997) had shown that in experiments with nonpeptide delta agonist BW373U86 and mu agonist fentanyl BW373U86 convulsive activity was attenuated by fentanyl and the fentanyl-induced

Straub tail effect was antagonized by BW373U86, and the hot-plate analgesic activity was additive between the two compounds, suggesting that complex inhibitory interactions take place between mu and delta receptors.

Several mechanisms could be responsible for the observed mu-delta opioid interaction that occurs at the behavioral level. Rossi et al. (1994) demonstrated that simultaneous activation of mu and delta opioid receptors from different brain regions at different sites within the nociceptive pathway resulted in a synergistic enhancement of analgesia. Activation of the receptors in the same brain region resulted in additive effects only. This suggests that the mu/delta opioid interaction occur between opioid receptors located in different pathways involved in the pain response instead of a direct receptor-receptor interaction (Rossi et al., 1994). Martin and Prather (2001) working with mu, delta, or both mu and delta opioid receptors stably transfected in rat pituitary GH3 cells, found that coexpression of mu and delta opioid receptors resulted in the appearance of multiple affinity states for mu but not delta opioid receptors. These results suggest that cotransfection of mu and delta opioid receptors alters the binding and functional characteristics of the receptors, and that the simultaneous exposure of GH3 cells cotransfected with mu and delta receptors to selective mu and delta opioid agonists produces an interaction between receptors resulting in enhanced receptor binding.

In vitro and *in vivo* studies performed on mu knock-out mice (Matthes et al., 1998) demonstrated partially reduced activity of the delta opioid receptor, but no changes were detected in the activity of the kappa receptors. Intact antinociceptive and respiratory responses to the kappa agonist suggest that the kappa receptor mainly acts independently from the mu receptor *in vivo*. Reduced delta analgesia and the absence of delta respiratory depression in mu opioid receptor-deficient mice together indicate that functional interactions may take place between mu receptors and central delta receptors in specific neuronal pathways.

Using different approaches to study the localization of mu and delta receptors it was shown that mu and delta receptors often colocalize (Arvidsson et al., 1995; Baumhaker et al., 1993; Palazzi et al., 1996). These results suggest that the existence of delta and mu receptor complexes is physically possible.

The existence of dimers and oligomers for many GPCRs mediating the actions of neurotransmitters is well documented (George et al., 2000). Early studies showing that dimeric analogs of oxymorphone and enkephalin exhibit higher affinity and potency than their monomeric forms suggested that mu receptors could function as dimers (Hazum et al., 1982). In 1997 Cvejic and Devi demonstrated that delta opioid receptors existed as homodimers and undergo agonist-mediated monomerization. Furthermore, it was shown that fully functional delta receptors heterodimerize with kappa receptors and heterodimerization affects their ligand binding and signaling properties (Jordan and Devi, 1999).

Recently Gomes et al. had examined whether heterodimerization of mu and delta receptors could account for the cross-modulation previously observed between these two receptors (2000). Their studies show that heterodimers exhibit distinct ligand binding and signaling characteristics. It was found that co-expression of mu and delta receptors in heterologous cells followed by selective immunoprecipitation results in the isolation of mu/delta heterodimers. Treatment of these cells with extremely low doses of delta selective ligands resulted in a significant increase in the binding of a mu receptor agonist. Similarly, treatment with mu selective ligands resulted in a significant increase in the binding of a delta receptor agonist. Furthermore, a delta receptor antagonist enhanced both the potency and efficacy of the mu receptor signaling; likewise a mu antagonist enhances the potency and efficacy of the delta receptor signaling. A combination of agonists (mu and delta receptor selective) also was demonstrated to synergistically bind and potentiate signaling by activating the mu-delta heterodimer.

Another group (George et al., 2000) provided evidence for the direct interaction of mu and delta opioid receptors to form oligomers and the generation of novel pharmacological and functional characteristics when mu and delta opioid receptors are expressed together. Each receptor expressed individually was pharmacologically distinct and could be visualized following electrophoresis as monomers, homodimers, homotetramers, and higher molecular mass oligomers. When mu and delta opioid receptors were coexpressed, selective non-peptide agonists for each had reduced potency and altered rank order, whereas endomorphin-1 and Leu-enkephalin had enhanced affinity, suggesting the formation of a novel binding pocket. Heterooligomers, but not

heterodimers, were identified in the cells coexpressing mu and delta receptors by immuno-precipitation. In contrast to the individually expressed mu and delta receptors, the coexpressed receptors showed insensitivity to pertussis toxin and continued signal transduction, likely due to interaction with a different subtype of G protein.

2. AIM OF THE STUDIES

Despite the big progress made in opioid research, further studies are clearly needed to understand the mechanisms of action of opioid receptors. The development of new, highly selective ligands and investigation of the reasons underlying their selectivity is one of the ways leading to the full comprehension of the problem.

Ligands that exert their action trough mu and delta opioid receptors have enormous importance for both basic research and clinical use. The aim of the present study was to test biochemically and functionally several newly developed ligands acting through mu and delta opioid receptors and to study their structure – activity relationships. The studied ligands included synthetic as well as modified endogenous compounds.

Derivatives of well-known non-peptide delta selective antagonists naltrindole and naltriben were synthesized and structure-activity relationships of newly developed **indolo-** and **benzofuromorphinans** were investigated. The aim was to improve the selectivity and/or affinity of the indolo- and benzofuromorphinans with focus on the 5- and 14-positions.

New structural analogues of highly potent synthetic compound **etorphine**, which exerts its effects largely through mu opioid receptor, were synthesized and examined. The goal was to investigate the effects of structural **β-substituted modifications** on biochemical and functional properties of the compounds.

The endogenous mu-opioid selective peptides **endomorphin 1** and **2** were investigated. The aim was 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.

The study was performed in rat brain membranes using *in vitro* radioligand binding experiments and the [^{35}S]GTPγS binding assays.

3. MATERIALS AND METHODS

3.1. Chemicals

3.1.1. Radiochemicals

Tritiated [^{3}H]ethylketocyclazocine ([^{3}H]EKC; 20 Ci/mmol) was purchased from Du Pont-New England Nuclear (Boston, MA, USA). [^{3}H][D-Ala^2(Me)Phe^{4}Gly^{5}ol]enkephalin ([^{3}H]DAMGO; 55 Ci/mmol) was from Amersham, (Amersham Place, Little Chalfont, UK). [^{3}H]naloxone (35 Ci/mmol) and [^{3}H][Ile^{5,6}]deltorphin II (49 Ci/mmol) were synthesized in the Isotope Laboratory of Biological Research Center, Szeged (Toth et al., 1982; Nevin et al., 1994). Guanosine-5'-[γ-^{35}S]-triphosphate (1204 Ci/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary).

3.1.2. Studied ligands

The indolo,- and benzofuromorphinans were prepared by Dr. H. Schmidhammer and colleagues (Institute of Organic and Pharmaceutical Chemistry, University of Innsbruck, Innsbruck, Austria). Etorphine derivatives were synthesized in the laboratory of Dr. S. Makleit (Department of Organic Chemistry, University of Debrecen, Debrecen, Hungary). Di-, tri- and tetrapeptide fragments of endomorphin 1 were synthesized by Dr. Cs. Tomboly and G. Toth in the Isotope Laboratory of the Biological Research Center (Szeged, Hungary), and endomorphin 2 derivatives were prepared by L. Kocsis and Dr. Gy. Orosz (Research Group of Peptide Chemistry, Eotvos University, Budapest, Hungary).

3.1.3. Other chemicals

All reagents were of analytical grade, purchased from Sigma Chemicals (St. Louis, MO, USA).

3.2. Animals

Wistar rats (250-300 g body weight) were from the Animal House of the Biological Research Center (Szeged, Hungary). Rats were housed in groups of four, allowed free access to food and water and maintained on a 12/12-h light/dark cycle. The animals were kept and treated according to the European Communities Council Directives (86/609/EEC) and the rules of the Committee for the Protection of Animals in Research (University of Szeged, Szeged, Hungary).

3.3. Brain membrane preparation

A crude membrane fraction of Wistar rat brains was prepared according to Benyhe et al. (1997). Briefly, the animals were decapitated, their brains, without cerebella, rapidly removed and homogenized in 30 volumes of ice-cold 50 mM Tris-HCl (pH 7.4) buffer by a teflon-glass homogeniser. After centrifugation at 40,000 × g for 20 min at 4°C the resulting pellets were suspended in 30 volumes of the same buffer and incubated for 30 min at 37°C to remove endogenous opioids. Centrifugation was then repeated as described above. The final pellets were suspended in 5 volumes of 50 mM Tris-HCl (pH 7.4) buffer containing 0.32 M sucrose and stored at –70°C. Membranes were thawed before use, diluted with buffer and centrifuged at 40,000 × g for 20 min at 4°C to remove sucrose. The resulting pellets were taken up in appropriate fresh buffer and immediately used.

3.4. Radioligand binding experiments

3.4.1. Theoretical basis

Radioligand binding is a straightforward technique that allows to measure the binding of a certain ligand, agonist or antagonist, to a receptor. There are two major types of radioligand binding experiments: direct and indirect. The difference between two techniques is whether the direct interaction of a radioligand with receptor is measured or indirect inhibition by unlabelled ligand of the binding of a radioligand. Radioligand binding assays can be used in a variety of preparations. This method allows to measure

the affinity of the drug towards its receptor rapidly and allows to quantify the number of receptor in the tissue. Most analyses of radioligand binding experiments are based on the law of mass action model, which describes ligand-receptor interactions as:

$$\text{Ligand} + \text{Receptor} \leftrightarrow \text{Ligand-Receptor}$$

At equilibrium, ligand-receptor complexes form at the same rate that they dissociate:

$$[\text{Ligand}][\text{Receptor}]K_{+1} = [\text{Ligand-Receptor}]K_{-1},$$

where K_{+1} is the association rate constant in units of $M^{-1}min^{-1}$

K_{-1} is the dissociation rate constant in units of min^{-1}

Equilibrium dissociation constant (K_d) is defined as

$$K_d = K_{-1}/K_{+1} = [\text{Ligand}][\text{Receptor}]/[\text{Ligand-Receptor}]$$

Thus, the equilibrium dissociation constant, K_d, expressed in units of moles/liter or molar, is the concentration of ligand, which, in case of a bimolecular, one-step reaction, occupies half of the receptors at equilibrium.

The concentration of unlabelled drug that results in radioligand binding halfway between the upper and lower plateaus is called the IC_{50} (inhibitory concentration 50%). The IC_{50} is the concentration of unlabelled drug that blocks half the specific binding.

The inhibitory constant (K_i) of the receptor for the competing drug is the concentration of the unlabelled drug that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors. It can be calculated from IC_{50} value using Cheng-Prusoff equation (1973):

$$K_i = IC_{50} / (1+([\text{Ligand}^*] / K_d),$$

where [Ligand*] is the concentration of the radioligand;

K_d is the equilibrium dissociation constant of the radioligand.

3.4.2. Competition (displacement) binding experiments

Competition binding assay is a type of assay in which the binding of the unlabelled compound to a receptor is measured by its ability to displace the specific

binding of a low fixed concentration of a radioligand. This technique is widely used and allows to determine the equilibrium inhibition constant (K_i) of the tested ligand.

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 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 fiber filters (Table 3.1.) 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.

Table 3.1. Assay conditions used in the binding experiments.

Radioligand	Concentration (nM)	Incubation Time	Incubation Temperature	Filter	Test tube
[^{3}H]naloxone	1.0	60 min	0°C	GF/B	glass
[^{3}H]DAMGO	0.5	45 min	35°C	GF/C	glass
[^{3}H][Ile^{5,6}]deltorphin II	0.5	45 min	35°C	GF/C	plastic
[^{3}H]EKC*	1.0	45 min	25°C	GF/C	glass

*in the presence of 100 nM DAMGO and 100 nM [D-Ala^2, Leu^5]enkephalin.

3.5. [^{35}S]GTPγS binding

Binding of an agonist to an opioid receptor changes its conformation, which leads to the subsequent activation of G proteins. GTPγS (guanosine 5'-O-(γ-thio)triphosphate) is a thiol derivative of GTP, that is resistant to hydrolysis by GTPase activity of G protein α subunits. The presence of the nonhydrolisable GTP analogue changes the G protein activation cascade, allowing to measure the amount of activation. To study the activation of G protein by agonist-bound receptors in a quantitative manner, the binding of

radiolabelled [^{35}S]GTPγS analogue (1000-1400 Ci/mmol) to G proteins is determined (Wieland and Jakobs 1994, Traynor and Nahorski 1995).

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 three independent experiments performed in triplicates.

3.6. Determination of protein concentration

The protein concentration was determined by the method of Bradford (Bradford, 1986), using bovine serum albumin as standard.

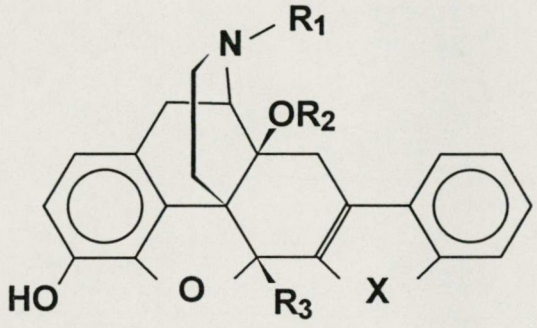
3.7. Data analysis

Obtained data were analyzed by Ligand (Munson and Rodbard, 1980), GraFit (Leatherbarrow, 1992) and GraphPad Prism (Stannard, 1995) computer programmes. Statistical analysis of the data was made by ANOVA. For significant ANOVA values, groups were compared by Dunnet's test. A probability level of $p < 0.05$ was accepted as indicating significant differences.

4. RESULTS AND DISCUSSION

4.1. 14-Alkoxy-Substituted Indolo- and Benzofuromorphinan Ligands

Non-peptide antagonists are of a big importance for scientific and clinical purposes because of their ability to penetrate blood brain barrier and stability against enzymatic degradation.



	R_1	R_2	R_3	X
NTI	CPM	H	H	NH
NTB	CPM	H	H	O
1	CPM	Me	Me	NH
2	CPM	Et	H	NH
3	CPM	Me	H	NH
4	Me	$CH_2CH_2CH_3$	Me	NH
5	CPM	CH_2Ph-3-NO_2	H	O
6	CPM	CH_2Ph-2,6-diCl	H	O
7	CPM	CH_2Ph-2-Cl	H	NH
8	CPM	2-naphtylmethyl	H	O
9	CPM	CH_2Ph-2-F	H	O
10	CPM	Et	H	O
11	allyl	allyl	H	N-allyl

Fig.4.1.1. Chemical structure of the tested compounds.

CPM = cyclopropylmethyl

The structure of the well-known delta opioid antagonists naltrindole (NTI) and naltriben (NTB) was modified and the structure-activity relationship of the newly developed indolo- and benzofuromorphinans was investigated. The chemical structures of the original and new compounds are shown in Fig. 4.1.1.

Since methylation at position 5 (substitution R_3) was reported to play an important role in decreasing mu antagonism and thus increasing delta selectivity, while a 14-EtO group (substitution O-R_2) in indolomorphinans was found to be somewhat superior to both a 14-MeO group and a 14-PrO group concerning delta antagonism (Schmidhammer et al., 1998), it was expected that new compounds will demonstrate improved selectivity and/or affinity of the indolo- and benzofuromorphinans with focus on the 5- and 14-positions.

Affinity and selectivity

To determine the binding characteristics of newly synthesized ligands, radioligand binding assays in rat brain membrane were carried out. In competition binding experiments increasing concentrations of the tested compounds were competing for the binding with well known type-selective tritiated ligands: [^{3}H]Ile^{5,6}Deltorphin II was used to characterize the binding to delta binding sites, [^{3}H]DAMGO and [^{3}H]EKC for mu and kappa binding sites, respectively.

All new compounds showed high affinity towards delta binding sites with K_i values in the subnanomolar or nanomolar range (Table 4.1.1). Affinities of the compounds towards mu and kappa binding sites were generally one to two orders of magnitude lower than for delta binding sites. Selectivity ratios determined as ratio K_i^{mu}/K_i^{delta} or K_i^{kappa}/K_i^{delta} showed general preferences of the ligands for delta binding sites.

Compounds **3**, **4**, **5**, **7**, **8**, **9** and **11** had comparatively low delta selectivity with affinities for delta binding sites in the nanomolar range. Compounds **1** and **2** had high delta selectivity, with slightly lower mu/delta ratios and higher kappa/delta ratios than those of NTI. The 14-ethoxy-substituted (R_2) benzofuromorphinan **10** showed higher affinity for delta binding sites compared to NTB, what resulted in 6.4 - fold increased mu/delta selectivity. Compound **6**, which has CH_2Ph-2,6-diCl group at R_2, showed a

decrease in both affinity and selectivity for delta binding sites as compared to NTB and demonstrated the lowest affinity and selectivity for delta opioid receptors in this group.

Compounds **1** and **2** had similar mu/delta ratios slightly less than the one of NTI and somewhat better delta/kappa selectivity than NTI. Compound **1** had general decrease of affinity towards all mu, delta and kappa binding sites, comparing to NTI, whereas **2** showed decreased affinity for kappa binding sites.

Table 4.1.1. Affinities and selectivity of tested compounds

	K_i, nM			Selectivity	
compound	μ	δ	κ	μ/δ	κ/δ
NTI	30.40±0.70	0.09±0.04	27.52±2.41	338	306
NTB*	80.8±2.3	0.54±0.09	-	150	-
1	283.55±25.27	1.15±0.85	420.52±141.27	247	366
2	96.90±34.80	0.40±0.08	313.00±98.90	242	783
3	68.70±11.10	1.44±0.40	103.00±21.60	48	72
4	241.98±22.21	9.02±3.62	218.89±68.08	27	24
5	419.00±42.80	10.90±1.24	373.00±125.00	38	34
6	158.00±9.68	30.80±4.03	64.90±5.02	5	2
7	164.79±1.54	8.00±0.31	130.50±13.16	21	16
8	53.30±11.10	4.60±1.16	350.00±123.00	12	76
9	138.52±18.66	9.90±4.36	148.66±25.22	14	15
10	116.00±12.40	0.12±0.05	253.00±65.10	967	2108
11	187.38±17.80	4.66±3.29	116.52±19.31	40	25

*Data are taken from Spetea et al, 1998a.

In compounds **2** and **3** the introduction of an alkoxy group at position 14 (O-R_2) changed the binding characteristics of ligands compared to parent NTI, resulting in decreased affinities of the ligands towards all types of opioid binding sites, especially in case of **3**. Selectivity of the compound **2** remained comparable with the selectivity of the parent NTI, but decreased for compound **3**, suggesting that introduction of Me at R_2 is not

favorable for delta selectivity. When H is changed for Me at position R_2 (ligand **3**), delta selectivity of the compound decreased because there was a dramatic loss of delta receptor affinity, but only small decrease in mu and kappa affinities.

In compound **7** the introduction of CH_2Ph-2-Cl group at position R_2 resulted in a loss of affinity of the ligand toward delta binding sites and a consequent decrease in selectivity. Analyzing ligands **5, 6, 8, 9, 10** and parent compound NTB, which differ only in substitution at R_2, the affinity of the ligands for delta binding sites changes in the following order: compound **10** (Et) > NTB (H) > **8** (2-naphtylmethyl) > **9** (CH_2Ph-2-F) ≈ **5** (CH_2Ph-3-NO_2) > **6** (CH_2Ph-2,6-diCl), and selectivity ranges: **10** >> NTB >> **5** ≈ **8** ≈ **9** > **6**. Comparison of the compounds **5** and **6** shows that presence of two Cl atoms of at CH_2Ph- chain introduced in position R_2 results in increase of affinity of the ligand **6** towards mu and kappa binding sites and decrease of affinity towards delta binding sites, thus leading to almost complete loss of selectivity.

When hydrogen is exchanged for methyl groups at R_2 and R_3, as in compound **1**, this leads to an approximately equal decrease of affinities of the ligand for all types of opioid receptors, compared to NTI.

Ligand **1**, which has Me incorporated at position 5 (R_3), demonstrated much better selectivity towards delta binding sites than compound **3**. This increase in selectivity appears from significant loss by compound **1** of the affinity for mu and kappa binding sites.

Introduction of allyl group at positions R_1, R_2 and X resulted in the compound **11**, which demonstrated moderate affinity and selectivity for delta binding sites.

Agonist/antagonist character

Agonist/antagonist properties of the new ligands were assessed using well known *in vitro* effect produced by Na^+ ions on opioid binding (Pert and Snyder, 1974) (Table 4.1.2). Inhibition of the binding of the general opioid antagonist [^{3}H]naloxone by various concentrations of tested compounds was measured in the presence and absence of 100 mM NaCl.

According to their Na^+ – indices, calculated as ratios $K_i(+Na^+)/K_i(-Na^+)$, all new ligands, except **4** were shown to be either pure antagonist (compounds **2, 3, 5, 6, 7, 9, 10,**

11 and **8**) or mixed agonist/antagonist (compound **1**). Ligand **4**, which has Me at R_1 showed agonistic features, with Na^+-index equal to 15.5. This observation is in a good agreement with the literature data, confirming the importance of N-substitution for agonist/antagonist characteristics of ligands. Parent compounds, NTI and NTB, are well known antagonists.

Table 4.1.2. Displacement of [^{3}H]naloxone by tested compounds

	K_i, [^{3}H]naloxone, nM		
compound	$-Na^+$	$+Na^+$	Na^+ index
NTI	15.17±1.20	6.90±0.28	0.5
NTB*	44	44	1
1	135.25±21.06	347.56±176.92	2.6
2	44.90±9.44	33.10±5.43	0.7
3	24.00±3.70	15.30±1.79	0.6
4	289.97±48.56	4488.27±2012.52	15.5
5	359.00±45.90	306.00±17.20	0.9
6	188.00±30.90	132.00±23.80	0.7
7	107.59±12.94	67.72±1.93	0.6
8	138.00±37.00	158.00±22.20	1.1
9	81.58±15.23	71.02±4.69	0.9
10	58.30±10.70	49.10±7.03	0.8
11	95.14±15.60	78.73±4.73	0.8

*Data are taken from Spetea et al., 1998a

Functional assays

[^{35}S]GTPγS binding assays were performed to evaluate the ability of newly synthesized compounds to stimulate the activity of G-proteins. Ligand **4**, which demonstrated agonistic character in radioligand displacement experiments, was able to stimulate [^{35}S]GTPγS binding in dose-dependent manner with EC_{50} = 497.2±138.9 nM (Fig. 4.1.2.).

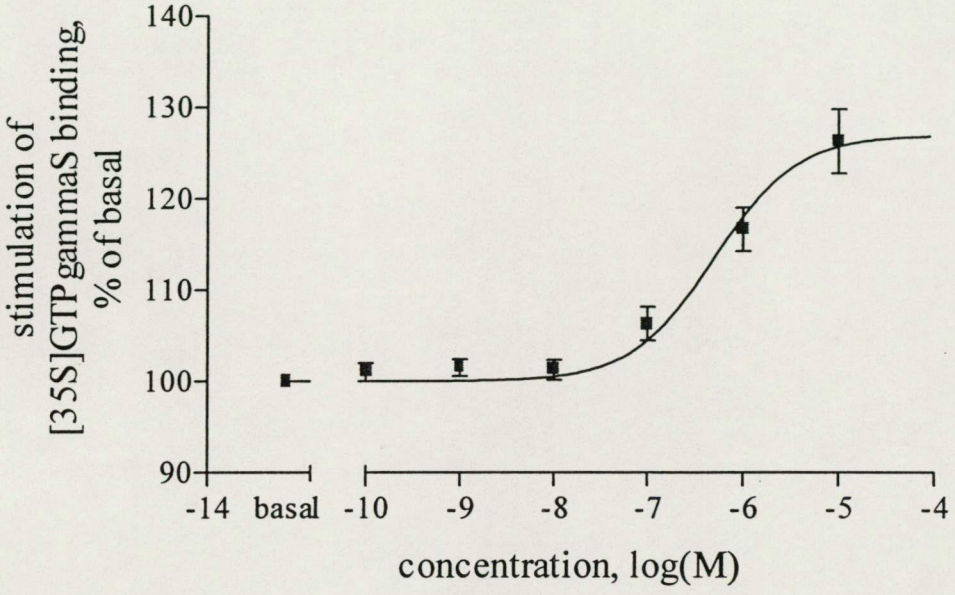


Fig. 4.1.2. Stimulation of [^{35}S]GTPγS binding by compound **4**. Non stimulated [^{35}S]GTPγS binding was 100.02±29.13 fmol/mg of protein (mean values ± SEM), non specific binding was 27±12 % (mean values ± SEM).

In accordance with their antagonist character, proved in radioligand binding assays, none of the tested compounds, except ligand **4**, stimulated [^{35}S]GTPγS binding (data not shown). When these ligands were evaluated in inhibiting the agonist response produced by the delta selective agonist Ile^{5,6}Deltorphin 2, all of them were able to decrease significantly the stimulatory effect produced by the peptide agonist ($p<0.05$ by Dunnett's test, Table 4.1.3.).

All the new compounds, except **4**, were demonstrated to have full antagonist profile in inhibiting of the Ile^{5,6}deltorphin 2 stimulated activation of G-proteins.

Table 4.1.3. Effect of the tested compounds on delta opioid agonist Ile^{5,6}deltorphin II - stimulated [^{35}S]GTPγS binding.

compound	% of stimulation/inhibition over basal activity
non-stimulated basal activity	100
Ile^{5,6}deltorphin II (δ selective opioid agonist)	117±1
Ile^{5,6}deltorphin II+**Nx** (general opioid antagonist)	102±4*
Ile^{5,6}deltorphin II+**NTI** (δ selective opioid antagonist)	97±3*
Ile^{5,6}deltorphin II+**1**	104±3*
Ile^{5,6}deltorphin II+**2**	103±3*
Ile^{5,6}deltorphin II+**3**	96±4*
Ile^{5,6}deltorphin II+**5**	94±5*
Ile^{5,6}deltorphin II+**6**	97±3*
Ile^{5,6}deltorphin II+**7**	103±2*
Ile^{5,6}deltorphin II+**8**	102±3*
Ile^{5,6}deltorphin II+**9**	95±3*
Ile^{5,6}deltorphin II+**10**	94±3*
Ile^{5,6}deltorphin II+**11**	100±5*

*Data are $p<0.05$ different from Ile^{5,6}deltorphin II alone, as determined by Dunnett's test.

* * *

Examination of the chemical structure, binding affinities and selectivities of the new developed opioid compounds reveals certain structure-activity relationships for the investigated compounds. First, the presence of a 5-Me group induced no change in delta affinity, but decreased the mu and kappa affinities, thus increasing mu/delta and kappa/delta selectivity ratios. Second, the substitution at position 14 is a very important determinant in increasing delta selectivity. An ethoxy group in this position confers a very high affinity and also high selectivity to delta opioid receptors. Chain prolongation of the 14-alkoxy group (compounds **4** and **11**) and introduction of benzyl- and

naphthylmethyl groups at the oxygen in position 14 (compounds **5-9**) resulted in compounds with reduced delta affinity and selectivity. A benzofuro moiety (compound **10**) seems to be superior to an indolo moiety (compound **2**) regarding both delta affinity and selectivity in this class of compounds. Indolic N-substitution (compound **11**) does not seem to have much influence on delta affinity and selectivity.

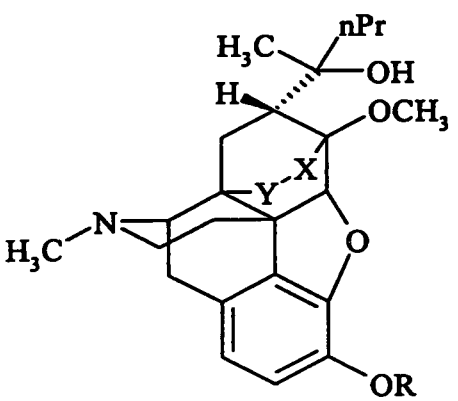
The pharmacological properties of the investigated compounds are correlated very well with their structure. It is well known that the nature of the substituent at the morphinan nitrogen is very important concerning opioid agonist/antagonist features of the compound. The delta selective agonist **4** possesses a methyl group at the 17-N position, whereas the antagonists NTI, NTB, **1-3** and **5-11** have "antagonist" substituents such as cyclopropylmethyl or allyl at this position.

The results of the study demonstrate that the positions 5 and 14 of indolo- and benzofuromorphinans represent critical sites that could be a trigger to develop new compounds with increased delta affinity and/or selectivity.

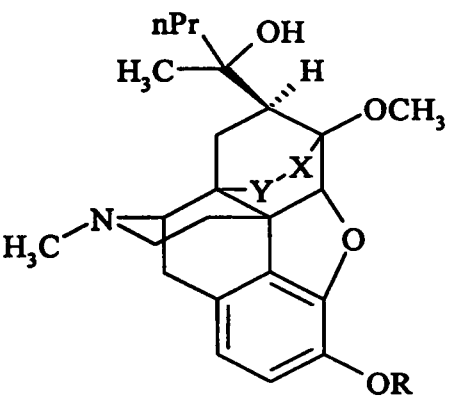
4.2. Etorphine derived ligands

Etorphine, compound with heterocyclic structure, is a highly potent synthetic opioid ligand, acting through all three types of opioid receptors with preference for mu binding sites (Melone et al., 2000). It is known to cause strong analgesia, catatonia and blockade of conditioned reflexes in laboratory animals (Lister R.E., 1966). In a veterinary and nature conservation practice etorphine is widely used for the immobilization of large animals (its trade name is Immobilon®). Though being very potent analgesic, etorphine is not used in medicine due to strong respiratory depression it causes in human.

Fig. 4.2.1. Structure of the α-, (1) and β-etorphine (2) derived compounds



1 a-d



2 a-d

1a: R = H, x-y = -CH=-CH- [7, 8]
1b: R = CH_3, x-y = -CH=-CH-
1c: R = H, x-y = $-CH_2-CH_2-$ [9]
1d: R = CH_3, x-y = $-CH_2-CH_2-$

2a: R = H, x-y = -CH=-CH-
2b: R = CH_3, x-y = -CH=-CH
2c: R = H, x-y = $-CH_2=-CH_2-$
2d: R = CH_3, x-y = $-CH_2=-C$

(nPr = propyl)

Etorphine played an important role in opioid receptor research. In fact, opioid receptors themselves were discovered in 1973 by demonstrating the specific binding of [^{3}H]etorphine to rat brain homogenate (Simon et al., 1973). Slowly dissociable oripavine radioligands [^{3}H]etorphine and [^{3}H]diprenorphine were successfully utilized in the

solubilization and purification procedures of opioid receptors (Simon et al., 1975) and helped also determining the molecular size of the native receptor-ligand complex in detergent extracts (Mollereau et al., 1988).

Though there is a number of structure-activity relationship studies dealing with etorphine derivatives (Katsumata et al., 1995; Wang et al., 1995; Lee et al., 1999), the biochemical and pharmacological properties of the β-substituted compounds (Bentley and Lewis, 1972) have not been studied until now.

Four new analogues of etorphine with C18-β configuration (Fig. 4.2.1) were constructed and their binding characteristics and ability to activate G-proteins studied. Corresponding α-etorphine derivatives were also tested for direct comparisons.

Radioligand binding experiments

Potencies of the newly synthesized compounds together with their corresponding α-etorphine derivatives in inhibiting the specific binding of the general opioid antagonist [^{3}H]naloxone to rat brain membranes were measured by equilibrium heterologous competition experiments. These studies also included the determination of the sodium shift (Pert and Snyder, 1974). Our results are summarized in Table 4.2.I., representative displacement curves of β-derivatives are shown in Fig. 4.2.2.

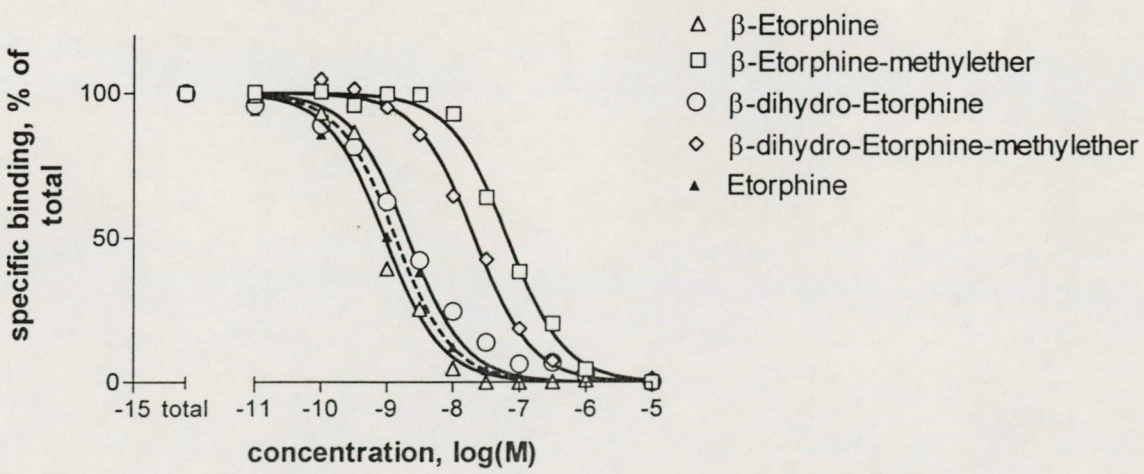


Fig. 4.2.2. Displacement of [^{3}H]naloxone by etorphine and its β-derivatives.

Etorphine derivatives inhibited the equilibrium binding of [^{3}H]naloxone with high affinity. β-etorphine demonstrated the highest ability to inhibit [^{3}H]naloxone binding, with K_i value of 0.47 ± 0.09 nM. Both α-, and β-dihydroetorphines had high affinity for opioid binding sites, though somewhat lower than parent α-, and β-etorphines. Methylation of the compounds significantly affected their ability to compete with [^{3}H]naloxone for the binding, increasing the K_i values. The rank order of the potencies of the tested compounds was β-etorphine ≥ etorphine ≥ dihydroetorphine ≥ β-dihydroetorphine > dihydroetorphine-methylether ≥ etorphine-methylether > β-dihydroetorphine-methylether > β-etorphine-methylether.

The effect of 100 mM NaCl was characterized by a decrease in the binding affinity. Significant increases of the K_i values in the presence of sodium ions suggested the agonist character of each compound tested (Table 4.2.1.).

Table 4.2.1. Displacement of [^{3}H]naloxone by tested compounds

Compound		K_i, nM		
		$-Na^+$	$+Na^+$	Na^+ index
Etorphine	**(1a)**	0.62 ± 0.08	4.26 ± 0.99	6.9
Etorphine-methylether	**(1b)**	4.54 ± 0.38	342 ± 183	75
Dihydroetorphine	**(1c)**	0.78 ± 0.39	13.1 ± 2.9	17
Dihydroetorphine-methylether	**(1d)**	3.58 ± 1.72	201 ±88	56
β-Etorphine	**(2a)**	0.47 ± 0.09	11.5 ± 1.3	24
β-Etorphine-methylether	**(2b)**	22.7 ± 1.5	1390 ± 918	61
β-Dihydroetorphine	**(2c)**	1.61 ± 0.34	13.8 ± 1.8	8.6
β-Dihydroetorphine-methylether	**(2c)**	10.5 ± 1.9	409 ± 51	39

Data represent means±S.E.M. of at least three independent experiments.

Functional assays

To investigate receptor-mediated activation of G-proteins by etorphines [^{35}S]GTPγS binding studies were performed. All tested compounds produced robust dose-dependent stimulation of [^{35}S]GTPγS binding (representative stimulation curves shown in Fig. 4.2.3., results are summarized in Tab.4.2.2.).

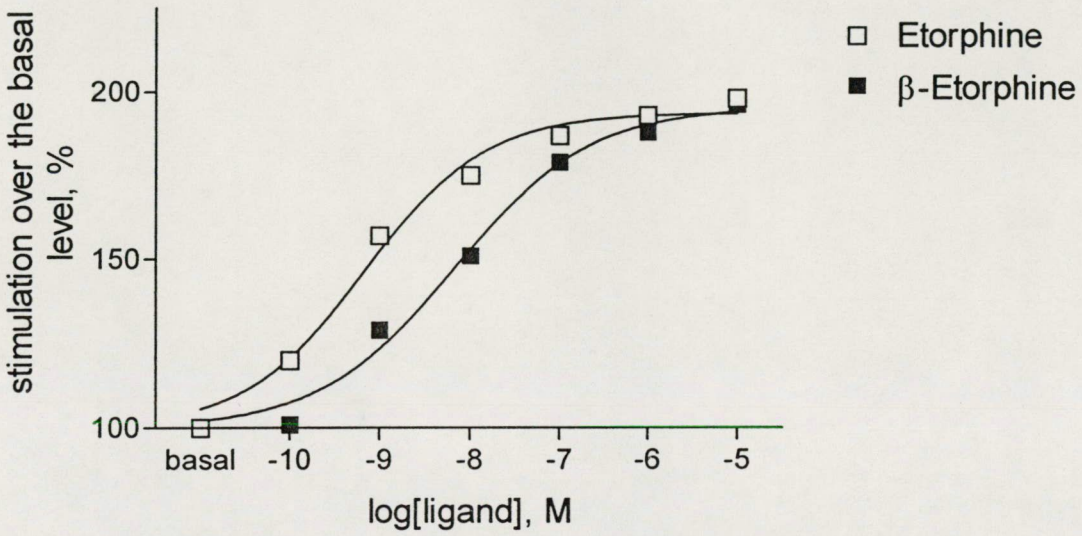


Fig. 4.2.3. Stimulation of [^{35}S]GTPγS binding by etorphine and β-etorphine.

This stimulation could be reversed by the addition of 10 μM naloxone, indicating the opioid nature of the process (Fig. 4.2.4, Table 4.2.2.).

The potencies of dihydro-compounds (both α-, and β-etorphines) in stimulating the [^{35}S]GTPγS binding were slightly higher than those of parent etorpnine compounds. Methylation of etorphine and dihydroetorphine (also for both α-, and β-structures) resulted in a loss of potencies of the compounds, shifting EC_{50} values into the high nanomolar range.

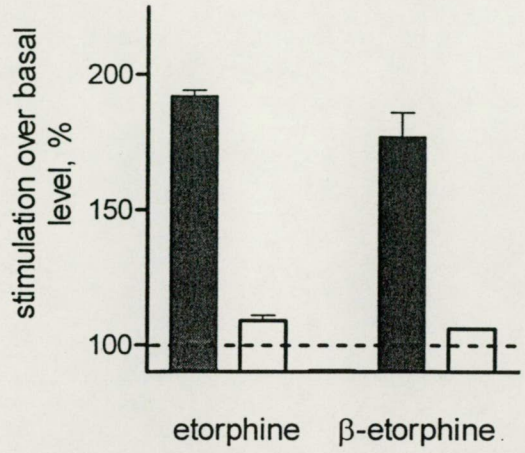


Fig. 4.2.4. [^{35}S]GTPγS binding stimulated by 1 μM etorphine or β-etorphine in the absence (filled bars) and presence (open bars) of 10 μM naloxone.

Table 4.2.2. Stimulation of [^{35}S]GTPγS binding by etorphine compounds

Compound		EC_{50},(nM)	Max. stimulation at 1 μM, % of the basal activity	
			-Nx	+Nx, 10 μM
Etorphine	**(1a)**	1.09±0.47	192±2	109±2
Etorphine-methylether	**(1b)**	405±99	156±2	101±3
Dihydroetorphine	**(1c)**	0.87±0.11	180±4	111±2
Dihydroetorphine-methylether	**(1d)**	113±19.7	165±2	102±1
β-Etorphine	**(2a)**	10.2±6.7	177±9	106±1
β-Etorphine-methylether	**(2b)**	675±126	159±7	100±2
β-Dihydroetorphine	**(2c)**	3.10±1.02	156±13	110±7
β-Dihydroetorphine-methylether	**(2d)**	205±27	168±9	102±1
No compounds added (basal level)			100	

* * *

From a structural point of view the currently presented β-etorphines are completely novel compounds, only β-buprenorphine was earlier constructed (Uff et al., 1985; Marton et al., 1995). β-Etorphines have similarly high affinity to opioid binding sites in comparison with the corresponding C18-α structures. O-Methylation at the C3 position consistently reduced the affinity of the ligands, whereas saturation of C7-C8 double bond resulting dihydro derivatives produced no substantial changes in the apparent affinity of the compounds. C3-methylation-dependent decrease in the affinity has long been obvious among the morphinan structures where methylation of morphine resulted in less effective analgesic compound codeine. Methylated etorphine and β-etorphine derivatives turned also to be less efficacious in functional studies.

Etorphine and dihydroetorphine derivatives behaved similarly in receptor binding assays and functional experiments, so the unique pharmacological features of dihydroetorphine, e.g., the low dependence potency (Qin, 1996; Tokuyama et al., 1993) can not be explained by simple structural reasons. Significant differences in the EC_{50} values between the phenolic compounds and methylether derivatives could reflect different efficacies of the compounds in activating heterotrimeric G-proteins in brain membranes.

Overall pharmacological properties of the drugs acting on the central nervous system receptors are dependent upon multiple factors, such as metabolism, lipid/water solubility changes, altered transport properties across the blood-brain barrier and different ability of ligand-induced receptor internalisation (Keith et al., 1998), etc. Similar factors may also be involved in the very high analgesic potency of etorphine-like compounds and in their reported capability of immobilising large animals (Osofsky, 1997).

The newly synthesized β-etorphine analogues have high affinities in [^{3}H]naloxone binding assays and they potently stimulate [^{35}S]GTPγS binding in neuronal membranes. Both radioligand binding and functional studies suggest the agonist nature of all the compounds tested. This biochemical predictions are in agreement with the chemical structure of the ligands and in several cases are further supported by pharmacological observations (Tokuyama et al., 1996; Aceto et al., 1997). Using etorphine and β-

etorphine series of ligands in binding and functional studies, it seems that opioid receptors are relatively insensitive in discriminating diastereoisomery at the C18 atom in the oripavine structure. The outstanding affinity and efficacy of etorphine and dihydroetorphine in ligand binding assays (Rosenbaum et al., 1984; Katsumata et al., 1995) and their high potency in pharmacological examinations render this compounds and the new β-structures to be valuable experimental tools in the opioid research and probably in medicine.

4.3. Endomorphin 1 and 2 derived ligands

Endomorphin 1 and 2 were discovered in 1997. As naturally occurring tetrapeptides with high affinity and selectivity for the mu opioid receptor, they offer an excellent opportunity to study the structure – activity relationship required for successful binding to mu opioid receptor and subsequent activation of G-proteins. Therefore, in an attempt to create new improved ligands based on endomorphin structure and to investigate whether shorter peptides sharing endomorphin sequence could efficiently bind to mu opioid receptors and trigger functional response, the products of possible enzymatic degradation of endomorphin 1 and C-terminal modified derivatives of endomorphin 2 were studied.

4.3.1. Effect of enzymatic degradation on binding and functional activity of endomorphin 1

Degradation of endomorphins occurs both *in vitro* (Peter et al., 1999) and *in vivo* (Hackler et al., 1997), even though the enzyme system involved in the *in vivo* degradation is not known. Carboxypeptidases Y or proteinase A can catalyse the hydrolysis of peptide amides and the splitting of the Trp^3-Phe^4 bonds (Peter et al., 1999; Berne et al., 1990). Aminopeptidase P, which is present in mammalian brain (Harbeck and Mentlein, 1991), could degrade N-terminal Xaa-Pro and generate tripeptides from endomorphins. Aminopeptidase M, on the other hand, recognises Xaa-Pro sequences and cleaves the peptide chain after this sequence (Xaa a hydrophobic amino acid) (Dua et al., 1985) by generating di-peptides. Therefore, di-, tri- and tetrapeptide fragments were synthesized to investigate the binding of degradation products of endomorphin 1 to mu opioid receptors, their ability to activate G-proteins and also to study if the degradation products might interfere with endomorphin 1-induced stimulation of G-proteins.

Radioligand binding experiments

To identify the consequences of the degradation of endomorpin 1 (Tyr-Pro-Trp-Phe-NH_2, E1-NH_2), theoretically possible di-, tri- and tetrapeptide acid (peptide fragments with carboxyl function at the C-terminal) and amides (peptide fragments with

amide function at the C-terminal) were synthesised and their effect on tritiated ligands binding was determined. Since endomorphin 1 has been shown to be mu opioid receptor specific, the well-known tritiated mu agonist [D-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin (DAMGO) and opioid antagonist [^{3}H]naloxone were used to test whether the peptide fragments of endomorphin 1 could interfere with tritiated ligands binding. For this purpose rat brain membranes were incubated with 1 nM [^{3}H]naloxone or [^{3}H]DAMGO and 10 μM tested peptides. From all the peptides only E1-OH, PWF-NH_2 and YPW-OH were able to inhibit more than 50% of the binding of tritiated ligands (Table 4.3.1.1.). The rest of the peptide fragments inhibited less than 30% of the tritiated ligand binding.

Table 4.3.1.1. Inhibition of [^{3}H]naloxone and [^{3}H]DAMGO binding by the peptide fragments. The table lists the remaining specific radioligand binding (in % of radioligand alone), after the addition of tested compound at 10 μM concentration.

	[^{3}H]naloxone	[^{3}H]DAMGO
Radioligand alone	100	100
Tyr-Pro-Trp-Phe-NH_2	0.0	0.0
Pro-Trp-Phe-NH_2	48.0±4.8	82.5±3.2
Trp-Phe-NH_2	63.8±9.6	91.8±1.5
Tyr-Pro-Trp-Phe-OH	10.7±1.8	4.9±1.3
Tyr-Pro-Trp-OH	33.7±6.4	36.1±4.4
Tyr-Pro-OH	77.7±3.1	82.7±6.2
Pro-Trp-OH	76.0±7.8	95.6±1.1

The peptide fragments, which could displace at least 50% of the radioligands from their binding sites, were further tested in competitive binding experiments, in which increasing concentrations of endomorphin 1 fragments were competing for the binding with tritiated naloxone or DAMGO (Table 4.3.1.2.). [^{3}H]naloxone and [^{3}H]DAMGO binding was inhibited by E1-OH with K_i values of two orders of magnitude higher than the parent E1-NH_2. The equilibrium inhibition constants measured for the tripeptide missing the C-terminal phenylalanine, YPW-OH, were low for both [^{3}H]DAMGO and

[^{3}H]Nx. The tripeptide amide without the amino terminal tyrosine, PWF-NH_2, also had a very low affinity for the [^{3}H]Nx. Because of the very small effect at 10 μM, the K_i value for [^{3}H]DAMGO binding was not determined for this peptide.

Table 4.3.1.2. Displacement of [^{3}H]DAMGO and [^{3}H]naloxone by tested peptides.

	K_i, nM	
	[^{3}H]DAMGO	[^{3}H]naloxone
Tyr-Pro-Trp-Phe-NH_2	1.57±0.27	7.76±1.18
Pro-Trp-Phe-NH_2	ND	3255±544
Tyr-Pro-Trp-Phe-OH	394±41	872±132
Tyr-Pro-Trp-OH	2111±541	3345±429

Data represent the average±S.E.M. of at least three independent experiments

ND = not determined

Functional assays

To measure the biological effects of the peptide fragments of E1-NH_2, the stimulation of [^{35}S]GTPγS binding to rat brain membranes was determined and compared with the effects exerted by E1-NH_2 and DAMGO (Fig. 4.3.1.). Both DAMGO and E1-NH_2 at 10 μM concentrations produced robust, concentration dependent (data not shown) stimulation of [^{35}S]GTPγS binding over basal level (maximal stimulation: 171±4% for DAMGO and 150±3% for E1-NH_2). By contrast, none of the peptide fragments of E1-NH_2 had significant stimulatory effect on [^{35}S]GTPγS binding at 10 μM ($p<0.05$, ANOVA), or even at 10^{-4} M (data not shown).

To check, whether any of the peptide fragments could affect the E1-NH_2-induced stimulation of [^{35}S]GTPγS binding, the E1-NH_2 and the degradation fragments were applied simultaneously. It was shown that the [^{35}S]GTPγS binding induced by sub-maximal concentration of E1-NH_2 (30 μM; stimulation: 135±4.1%), could be inhibited by 100 μM YPW-OH, while E1-OH and PWF-NH_2 at the same concentration had no effects (Table 4.3.1.2.).

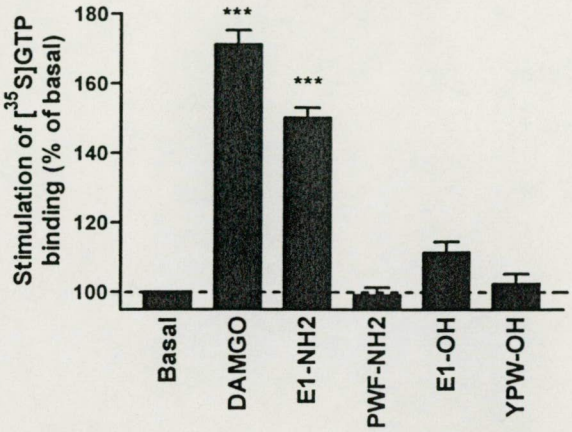


Fig. 4.3.1.1. Stimulation of [^{35}S]GTPγS binding to rat brain membranes by 10 μM E1-NH_2 or its peptide fragments. ***: values, significantly different from basal [^{35}S]GTPγS binding ($p>0.01$). Data represent the average±SEM of 3-5 independent experiments.

Table 4.3.1.2. Effect produced by the peptide fragments (100 μM) on E1-NH_2 (30 μM) induced [^{35}S]GTPγS binding.

compound	[^{35}S]GTPγS binding (% of basal)
Tyr-Pro-Trp-Phe-NH_2	135.2±4.1
Tyr-Pro-Trp-Phe-NH_2 + Pro-Trp-Phe-NH_2	136.0±3.2
Tyr-Pro-Trp-Phe-NH_2 + Tyr-Pro-Trp-Phe-OH	137.1±5.4
Tyr-Pro-Trp-Phe-NH_2 + Tyr-Pro-Trp-OH	111.4±2.7

* * *

Degradation of E1-NH_2 has been shown to occur both *in vivo* and *in vitro* (Peter et al., 1999; Hackler et al., 1997). Because of structural similarities to the parent compound, degradation peptides could interfere with the receptor binding properties of E1-NH_2 influencing its biological activity by binding directly to the receptor molecule or decreasing the optimal sterical conditions for E1-NH_2 binding. However, we found that

degradation turns E1-NH_2 into peptide fragments that possess low affinity to mu opioid receptors and do not activate G-proteins.

According to the "message – address" concept, Tyr^1 in E1-NH_2 can be viewed as the "message", since the tyramine moiety in opiate structures is known to be important for activity (Horn and Rodgers, 1977). It is not surprising, therefore, that the lack of this amino acid from PWF-NH_2 produced a several orders of magnitude decrease in the potency in displacing bound [^{3}H]Nx or [^{3}H]DAMGO. The address element in mu receptor recognition is the aromatic ring of Trp^3 as substitution of this site with non-aromatic amino acids lead to loss of recognition and mu specificity of E1-NH_2 (Paterlini et al., 2000). The presence of Trp^3 in WF-NH_2, however, was not sufficient for binding to rat brain membrane receptors suggesting that both pharmacophores (Tyr^1 and Trp^3) are necessary for binding to mu opioid receptor.

Not only the N-, but the C-terminal residues plays a critical role in the biological activity of E1-NH_2. The C-terminal amide group confers the high affinity of the binding of E1-NH_2. Modification of E1-NH_2 into E1-OH, the peptide acid derivative of E1-NH_2, resulted in a compound with a much lower affinity for the binding sites labelled by the radioligands used. The K_i values measured with E1-NH_2 and E1-OH differed in two orders of magnitude. Despite of its decreased ability to displace [^{3}H]DAMGO and [^{3}H]Nx, according to its Na^+ index E1-OH remained an agonist (data not shown). Furthermore, after the cleavage of Phe^4, the tripeptide YPW-OH displaced [^{3}H]DAMGO or [^{3}H]Nx binding with a more than three orders of magnitude lower affinity than E1-NH_2 did, highlighting the importance of not just the amino group, but also the fourth residue in binding E1-NH_2 to its receptor. In fact, it has been shown that a tetrapeptide with Gly^4 at its C-terminal, instead of Phe^4 like in E1-NH_2, had a decreased affinity to bind to mu receptor (Harrison et al., 1998) supporting our finding. Further cleavage of amino acids from the C-terminal completely abolished the mu receptor binding of the remaining dipeptide.

Despite the fact that E1-OH and the N- and C-terminal peptide fragments were able to displace some of the radioligand binding, most of these peptides had no effect on the activity of G-proteins *in vitro*. As was shown by *in vivo* experiments performed by M. Macsai at the Department of Pathophysiology of Albert Szent-Györgyi Medical Centre,

University of Szeged (Szatmari et al., 2001), none of these peptide fragments were able to induce analgesia in living animals. However, YPW-OH, the C terminal truncated tripeptide in large access could inhibit the E1-NH_2 induced stimulation of [^{35}S]GTPγS binding. It has been shown that E1-OH and PWF-NH_2 can be purified from brain (Hackler et al., 1997), but it remains to be elucidated whether YPW-OH does exist *in vivo*. It is also a question whether the concentration of this tripeptide could reach the needed concentration to exert its inhibitory effect. However, as YPW-OH could be generated by degradation of E1-NH_2 and other endogenous opioid peptides like hemorphin (Brant et al., 1986) or Tyr-W-MIF-1 (Erchegyi et al., 1992), this large concentration difference may occur, increasing the probability of physiological relevance of such inhibition.

The obtained results show that degradation of E1-NH_2 will largely destroy the biological activity of E1-NH_2, suggesting that Tyr-Pro-Trp-Phe-NH_2 represents a minimal sequence for mu opioid receptor recognition and specificity and its potent analgesic action. However, due to the ability of Tyr-Pro-Trp-OH to displace an amount of the E1-NH_2 binding, it is possible that this degradation peptide might be competitor for the binding site of E1-NH_2. Therefore, the decreasing effect of E1-NH_2 may take place not only because of degradation of the peptide or desensitization/down-regulation of mu receptors (Harrison et al., 2000), but because of sterical inhibition by the gradually increasing degradation product.

4.3.2. Effect of C-terminal modifications on binding and functional activity of endomorphin 2

The structural features of a ligand determine its binding characteristics and biological effects, so the modifications of amino acids may have profound effects on receptor selectivity and physiological action. In fact, DAMGO was derived from the original sequence of enkephalins (Handa et al., 1981) following the observations that N-methylation of the amino group of a phenylalanine[4] and alteration of the carboxyl group

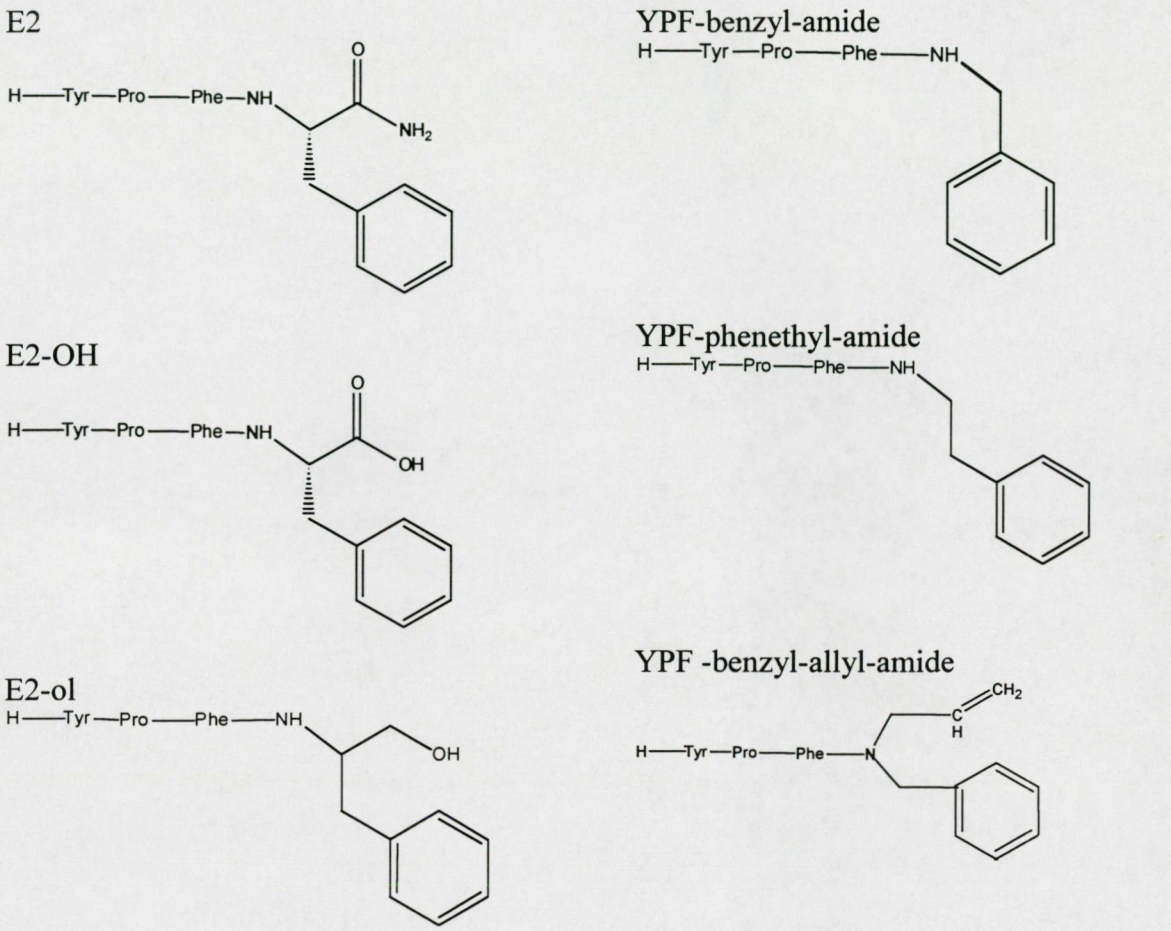


Fig. 4.3.2.1. Schematic structure of the endomorphin 2 and its derivatives

to an alcohol and replacement of the last amino acid (methionine[5]) to ethanolamine (glycinol) dramatically increased the analgesic activity and receptor binding affinity to a naloxone sensitive opiate receptor (Roemer et al., 1977; Kosterlitz et al., 1981). Based on these previous successful modifications and on the results of the experiments described in the section 4.3.1. the structure of endomorphin 2 (E2) was altered on its C-terminal in an attempt to increase its biological activity and to further our understanding of how structural modifications might influence the binding properties. Among the tested compounds (Fig. 4.3.2.1.) were the C-terminal acid derivative of endomorphin 2 (Tyr-Pro-Phe-Phe-OH, E2-OH) and ligands in which the carboxamide group of E2 was substituted by hydroxymethyl (E2-ol), allyl (Tyr-Pro-Phe-benzyl-allyl-amide), and by hydrogen (Tyr-Pro-Phe-phenethyl-amide) to test the importance of hydrogen bonding and electron-donor-acceptor properties at the C-terminal position.

Radioligand binding experiments

The ability of the endomorphin 2-derived peptides to bind to mu opioid receptor was assessed using tritiated endomorphin 2 ([^{3}H]E2) (Spetea et al., 1998b) and tritiated naloxone ([^{3}H]Nx) in competitive radioligand binding. The affinity of the different E2 derivatives towards mu binding sites was compared to that of DAMGO and E2. All peptides were able to displace tritiated E2 binding (Fig 4.3.2.2., Table 4.3.2.1.). E2-ol had an IC_{50} similar to DAMGO (0.70±0.17 nM and 0.98±0.49 nM respectively) while E2, the parent compound, was less potent in displacing [^{3}H]E2 (IC_{50} equal to 4.03±0.33). Apart from E2-ol, the C-terminally modified peptides were considerably less potent in inhibiting the binding of [^{3}H]E2.

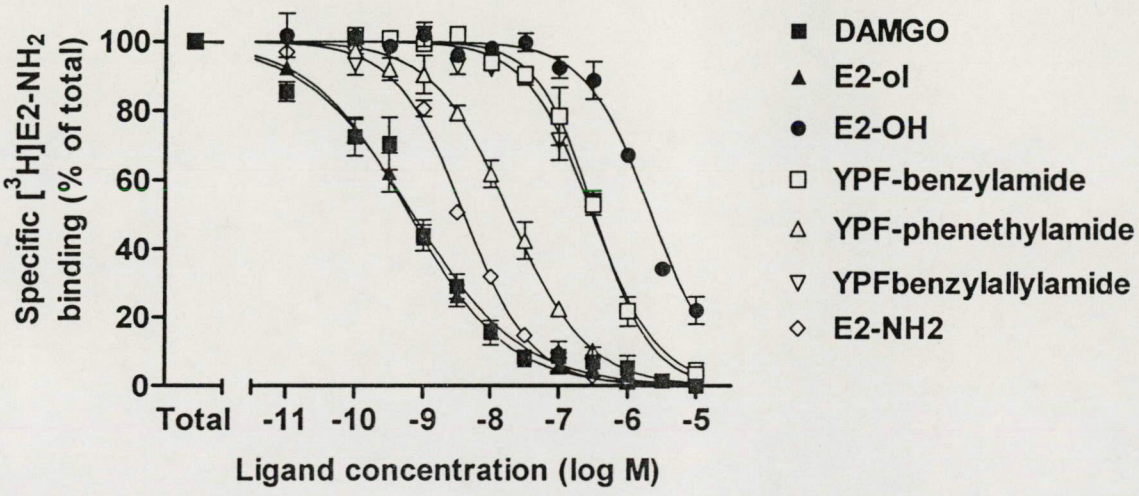


Fig. 4.3.2.2. Displacement of [^{3}H]endomorphin 2 by DAMGO, endomorphin 2 and its derivatives.

The displacement curves were shallow for DAMGO and E2-ol as indicated by their Hill coefficients (n_H), (0.53, 0.6 and 0.56 respectively), while the rest of the peptides had a n_H close to unity (data not shown). These results suggest that the amide-alcohol substitution changed the way the ligand interacts with the receptor.

Table 4.3.2.1. Displacement of tritiated endomorphin 2 and naloxone by endomorphin 2 derivatives.

	IC_{50}, nM			
	[^{3}H]endomorphin 2	[^{3}H]naloxone		
		-Na^+	+Na^+	Na^+ index
DAMGO	0.98±0.49	3.42±0.92	1030±354	300
endomorphin 2	4.03±0.33	8.15±1.61	736±179	90
endomorphin 2-OH	1820.0±458.0	>10000	>10000	-
endomorphin 2-ol	0.70±0.17	56.2±12.3	471±156	8
YPF-benzyl-amide	326.0±14.6	871±80.2	>10000	17
YPF-phenethyl-amide	18.50±3.73	264±30.3	4440±356	16
YPF-benzyl-allyl-amide	314.0±58.6	884±63.1	2070±171	2

[^{3}H]Nx binding was inhibited by E2 in the low nanomolar concentration range while E2-ol was less potent in displacing this ligand. The rest of the derivatives had >100 nM affinities for the [^{3}H]Nx binding site (Table 4.3.2.1.). The [^{3}H]Nx binding assay was repeated in the presence of 100 mM NaCl to investigate the sodium shift. Inclusion of 100 mM NaCl inhibited ligand binding to different extent, producing a robust decrease of affinity in case of E2 and DAMGO and more than 10-fold decrease for YPF-benzyl-amide and YPF-phenethyl-amide. Less than 10-fold shift was observed in the case of E2-ol and YPF-benzyl-allyl-amide.

Functional assays

The stimulation of [^{35}S]GTPγS binding by the structurally modified E2 derivatives was measured and compared to the effect exerted by E2 and DAMGO (Fig. 4.3.2.3.). The E2 stimulated maximal [^{35}S]GTPγS binding (154 %) was approximately 70 % of the DAMGO stimulated maximal activity (181 %). The maximal stimulation of [^{35}S]GTPγS binding by E2-ol, YPF-phenethyl-amide or YPF-benzyl-amide (131, 116 and 117 %, respectively) were all below the E2 or DAMGO stimulated maximum. Despite

inhibiting both [^{3}H]E2 and [^{3}H]Nx binding in competition experiments, YPF-benzyl-allyl-amide, E2-OH and YPF-OH had no agonist properties in this assay.

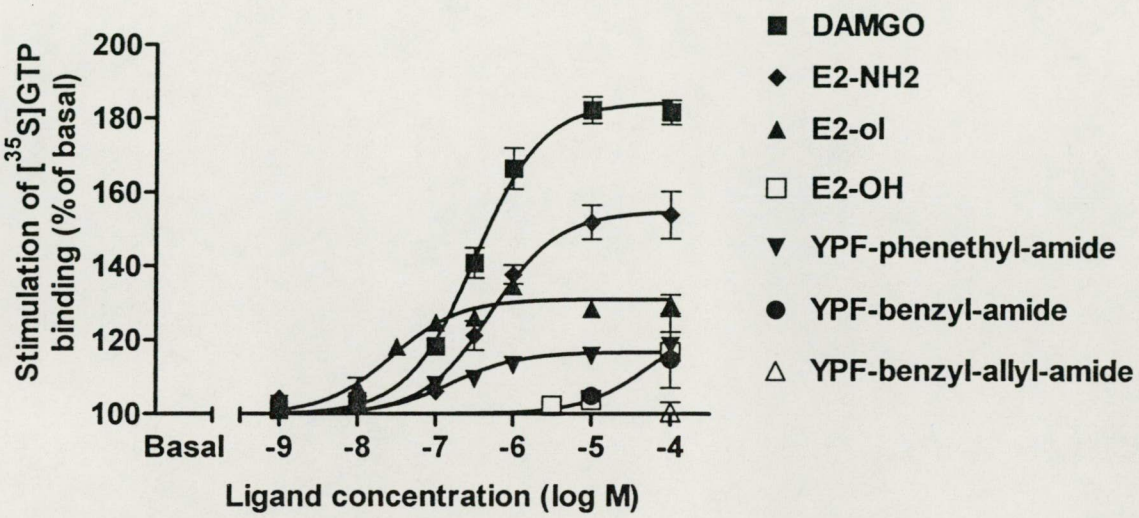


Fig. 4.3.2.3. Stimulation of [^{35}S]GTPγS binding by the derivatives of endomorphin 2.

Table 4.3.2.2. Stimulation of [^{35}S]GTPγS binding by endomorphin 2 derivatives

	EC_{50}, nM
DAMGO	347±40
endomorphin 2	572±97
endomorphin 2-OH	ND
endomorphin 2-ol	26.2±4.9
YPF-benzyl-amide	>1000
YPF-phenethyl-amide	178±103
YPF-benzyl-allyl-amide	ND

Data are means±S.E.M. ND = not determined

The stimulation of [^{35}S]GTPγS binding by the ligands investigated was concentration dependent. The most potent activator was E2-ol (EC_{50} equal to 26.2±4.9 nM), while the potency of E2 or DAMGO (EC_{50}: 572±97 nM and 347±40 nM, respectively) were one order of magnitude lower. YPF-phenethyl-amide had an EC_{50} of

178±103 nM, comparable to the potencies of E2 and DAMGO, while the EC_{50} for YPF-benzyl-amide was in the μM range.

It was checked whether some of the ligands will be able to at least partially inhibit the E2 or DAMGO stimulated [^{35}S]GTPγS binding. Indeed, the 1 μM E2 or DAMGO stimulated [^{35}S]GTPγS binding could be suppressed by 100 μM E2-ol, YPF-phenethyl-amide and YPF-benzyl-amide (Fig 4.3.2.3.) to the levels produced by the derivatives themselves.

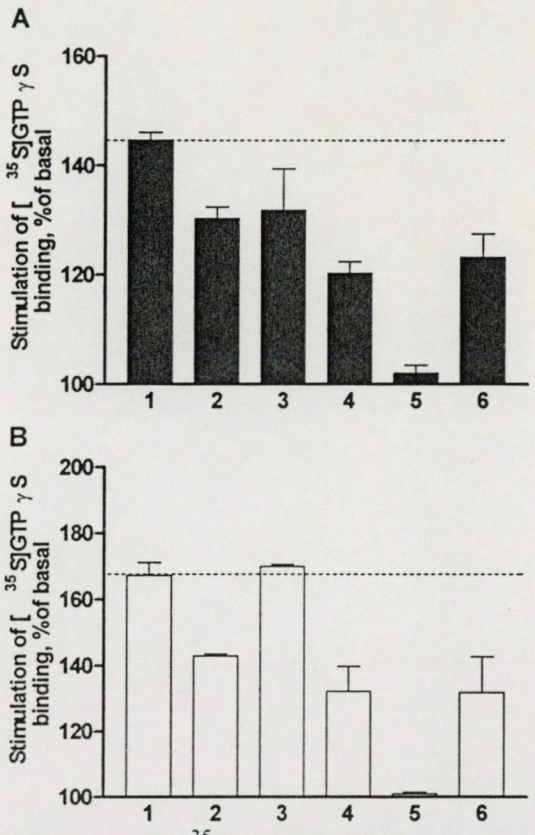


Fig. 4.3.2.3. Inhibition of [^{35}S]GTPγS binding stimulated by 1 μM of (**A**) endomorphin 2, and (B) DAMGO by the endomorphin 2 derivatives. The numbers represent: 1. ligand alone; 2. ligand + E2-ol; 3. ligand + E2-OH; 4. ligand + YPF-phenethyl-amide; 5. ligand + YPF-benzyl-allyl-amide; 6. ligand + YPF-benzyl-amide. Data represent the average ± S.E.M. of at least 3 independent experiments.

YPF-benzyl-allyl-amide fully inhibited both E2 or DAMGO stimulated [^{35}S]GTPγS binding. This inhibition was concentration dependent, but weak compared to the inhibition produced by naloxone (data not shown).

* * *

C-terminal residues play a critical role in the biological activity of mu opioid receptor specific ligands (Ronai et al., 1979). In fact, the C-terminal amino acid is essential for the high affinity binding of endomorphins to mu binding site (Harrison et al., 1998; Zadina et al., 1997; Szatmari et al., 2001). Because of the three aromatic side chains, endomorphins – despite of their small size - are relatively well structured (Fiori et al., 1999). However, the last amino acid, Phe^4, is free to adopt a "bio-active" conformation that is independent of the correct orientation or the stereochemistry of this residue (Paterlini et al., 2000).

Comparing of YPF-benzyl-amide and YPF-phenethyl-amide shows that a simple -CH_2 deletion results in one order of magnitude loss in receptor binding affinity and more than two orders of magnitude decrease in [^{35}S]GTPγS binding. This demonstrates that beside the conformational freedom, the spacing between the amide bond and the phenyl ring is also essential to obtain optimal binding and [^{35}S]GTPγS stimulation.

After replacing the amide group with an alcohol, E2-ol became as potent in displacing [^{3}H]E2 from its binding site as DAMGO. Furthermore, it was even more potent in replacing [^{3}H]E2 than E2 itself. This improved binding affinity might be the result of the C-terminal hydroxyl improving the otherwise very similar solution structure of E2 and DAMGO residue (Paterlini et al., 2000). Despite the high affinity binding, the maximal biological effect produced by E2-ol was only about one third of the effect produced by DAMGO, and about one half of that of E2. Based on the Na^+-index of E2-ol, the lower intrinsic activity could have been the result of becoming a partial agonist for both E2 and DAMGO induced [^{35}S]GTPγS binding. This hypothesis is supported by experiments on guinea pig ileum and mouse vas deferens (Al-Khrasani et al., 2001). Therefore, instead of producing a more potent agonist, replacement of -$CONH_2$ with -CH_2OH produced a high affinity partial agonist.

While E2-ol became a high affinity and low efficacy analogue of E2, most C-terminal modifications decreased the binding affinity and the functional activity of E2, producing partial agonists with varying degrees of inhibition. Modification of E2 to E2-OH dramatically decreased the binding of the tetrapeptide to mu opioid receptor, especially in the presence of Na^+, which was accompanied by a complete loss of biological activity, a similar result was described earlier for E1-OH.

It is also important to note that YPF phenethyl-amide performs well both in receptor binding and [^{35}S]GTPγS assays indicating that the C-terminal carboxamide or hydroxymethyl group is not essential for binding or G-protein activation. However, such groups are essential for achieving high level of stimulation. Replacement of phenylalanine amide to benzyl-allyl amide not just decreased the binding affinity by two orders of magnitude and transformed E2 into a partial agonist, but completely abolished its functional activity. This new compound became a weak but full, probably allosteric, inhibitor of E2 or DAMGO induced [^{35}S]GTPγS binding.

Inhibition of [^{3}H]Nx binding was also affected by the introduced modifications, but the change in binding affinity followed a different pattern. The binding affinities to the [^{3}H]Nx binding site were all lower than the parent compound, suggesting that the interactions (and might be the place of the receptor-ligand interaction on the receptor surface) of E2 and Nx with mu binding site is different. This is not an unexpected result as it has been shown that the interaction of agonists and antagonists with opiate receptors are different (Chaturvedi et al., 2000) (Law and Loh, 1999) (Kong et al., 1994).

In summary, it was demonstrated that alteration of the C-terminal carboxamide group mostly decrease the binding affinity and biological activity of 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 and [^{35}S]GTPγS binding. 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.

5. CONCLUSIONS

This study describes the results of *in vitro* biochemical characterization of several newly developed peptide and non-peptide compounds, acting on mu and delta opioid receptors. Radioligand binding experiments were performed to assess the binding characteristics of the compounds, and functional ([^{35}S]GTPγS binding) assays were used to investigate the ability of the new ligands to stimulate G-proteins. All experiments were performed in the rat brain membranes.

Several new indolo- and benzofuromorphinans substituted in the positions 5 and 14 were examined. All 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.

β-Substituted modifications of potent synthetic compound etorphine and its derivatives were studied. All new synthesized compounds showed high affinity for mu opioid receptors, though β-etorphines had slightly lower affinities compared to the 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 favourable 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.

To investigate how degradation of endomorphin 1 might influence ligand binding to the mu opioid receptor and consequent activation of G-proteins, a number of both N- and C-terminal truncated endomorphin 1-derived peptides was studied. The resulted peptides demonstrated considerably lower opioid receptor binding potency. None of these peptides had an effect on GTP binding. Obtained results suggest that degradation destroys the biological activity of endomorphin 1.

C-terminal modifications were introduced to endomorphin 2 to improve its binding properties and biological activity and to further our understanding of how structural modifications might influence the binding and functional properties. The alteration of the C-terminal carboxamide group mostly decreased the affinity of the peptides to the mu opioid receptor and their functional activity. 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 essential role in the regulation of the binding and the agonist/antagonist properties of E2.

The ligands described in this thesis represent potentially useful tools for opioid research. Further *in vivo* experiments would be important for studying the pharmacological properties of the compounds.

6. ÖSSZEFOGLALÁS

A természetes opioid peptidekkel, ópium-alkaloidákkal és a szintetikus opioid ligandokkal specifikusan kölcsönható sejtmembrán fehérjéket opioid receptoroknak nevezzük. Az opioid receptorok a hét helikális transzmembrán szegmenst tartalmazó (7TM), guanin-nukleotid kötő fehérjékhez kapcsolt (GPC) receptorcsalád tagjai. Az opioid agonisták kötődése a receptor fehérjéhez olyan jelátviteli eseményeket indít el, amelyek egyebek között a fájdalomérzet csökkenésének vagy például a morfin-függőség kialakulásának celluláris-molekuláris alapjait jelentik. Az opioid receptorok heterogén struktúrák, jelenleg három fő receptor típus, a morfin-, vagy μ-opioid receptor, az enkefalin-, vagy δ-receptor, valamint a dinorfin-, más néven κ-receptor elsődleges szerkezetét ismerjük. Az egyes receptor típusokra, illetve a még kevéssé ismert receptor altípusokra specifikus, nagy affinitású új ligandok kifejlesztése az opiátokkal kapcsolatos alap- és alkalmazott kutatások egyik jelentős tématerülete. Az újonnan előállított szelektív ligandok és radioligandok ugyanis a biokémiai és a farmakológiai kutatások elengedhetetlen eszközei, mindemellet pedig a hatékony fájdalomcsillapításban, a drogfüggőség kezelésében, megelőzésében, vagy más betegségekben hasznosítható gyógyszerek fejlesztésének kiindulópontjai lehetnek.

A disszertációban ismertetett kísérleti munka során μ- és δ receptorokon ható, új, eredeti, peptid szerkezetű és heterociklusos alkaloid-vázas ligandok *in vitro* biokémiai jellemzését végeztük el. A ligandok affinitását és szelektivitását radioligand kötési módszerekkel határoztuk meg patkány agyi sejtmembrán frakcióban. A ligandok receptor-közvetített G-protein aktiváló, vagy gátló hatásait funkcionális biokémiai teszttel, a [^{35}S]GTPγS kötés stimulálásával követtük nyomon az agyi membránpreparátumban.

A heterociklusos ligandok köréből több, 5-ös és 14-es pozícióban szubsztituált indolo- és benzofuranomorfinan származékot vizsgáltunk. Az összes analóg nagy δ-receptor affinitást mutatott. A piperidin nitrogén atomon (N-17) metil csoportot hordozó származék agonistának bizonyult a funkcionális biokémiai tesztben, megerősítve ezen molekularészlet kritikus szerepét az agonizmus / antagonizmus kérdésében. A nagyobb térkitöltésű N-17 szubsztituenseket hordozó ligandok mindannyian *in vitro* antagonitának mutatkoztak. Az 5-ös szénatom metilálása a fenti szerkezetekben nem változtatta meg a δ-receptor affinitást, azonban jelentősen

csökkentette a ligandok κ és μ kötőhelyek felé mutatott affinitását. Etoxi csoport bevitele a 14-es pozícióban mind a δ-affinitást, mind a δ-szelektivitást jelentősen megnövelte. Nagyobb lánchosszúságú 14-alkoxi csoport jelenléte viszont már csökkentette a δ-szelektivitást és affinitást. Eredményeink arra utalnak, hogy a hatékony, alkaloid vázas δ-szelektív opioid agonisták és antagonisták tervezése és fejlesztése során az 5-ös és 14-es pozícióban szubsztituált benzo- és indolofuranomorfinan analógok hatékony molekulák lehetnek.

A morfin analgetikus hatásait sokszorosan felülmúló szintetikus etorfin analógok, közöttük korábban már szintetizált, azonban biokémiai és farmakológiai kísérletekben még nem tanulmányozott β-etorfin származékok széleskörű biokémiai jellemzését is elvégeztük. A β-térállású analógokat minden esetben α-kiralitású izomerjeikkel hasonlítottuk össze. A vizsgált etorfin lingandok valamennyien igen hatékonynak bizonyultak μ-opioid receptor kompetíciós tesztekben, de a β-etorfinok valamivel kisebb affinitást mutattak az α-származékokhoz képest. Az etorfinok 3-OMe (metiléter) analógjai kisebb affinitással kötődtek a receptorhoz, mint a megfelelő 3-OH (fenolos) származékok. Hasonló hatás-szerkezeti összefüggések ismertek a morfin-vázas molekulák körében is: a morfin jobb analgetikum és nagyobb affinitású receptor ligand, mint metiléter származéka, a kodein. A C3-as helyzetben fenolos hidroxil csoportot tartalmazó vegyületeket G-protein aktivációs kísérletekben is hatékonyabbnak találtuk a metiléter analógokhoz képest. A részlegesen telített gyűrűszerkezetű dihidroetorfin és β-dihidroetorfin ligandokkal a C7-C8 kettős kötést tartalmazó etorfinhoz és β-etorfinhoz hasonló nagy affinitású receptor kötődést mutattunk ki. Eredményeinkből arra következtethetünk, hogy a 18-as szénatom α vagy β konfigurációja, illetve a C7-C8 kötés telítettségi szintje nem befolyásolja lényegesen az oripavin ligandok biológiai aktivitását.

Az opioid peptidek köréből munkánk során a közelmúltban felfedezett, kimagasló μ-receptor affinitással rendelkező endomorfin tetrapeptideket és struktúranalógjaikat tanulmányoztuk. Mivel az endogén opioid peptidek hatásainak terminációjában az enzimatikus degradáció (peptidáz aktivitás) folyamata és a hidrolízis peptidfragment-termékei fontos szerepet tölthetnek be, szisztematikus kísérletekben vizsgáltuk az endomorfin-1 peptid szintetikusan előállított tri- és dipeptid fragmenseinek, mint lehetséges degradációs termékeknek biológiai hatásait. A Tyr-Pro-Trp-Phe-NH_2 szerkezetű endomorfin-1 N és C-terminális felőli

"rövidítése", ami az aminopeptidázok, illetve a karboxi-peptidáz enzimek hatásainak eredményeit modellezte a peptid-fragment termékek szempontjából, markáns hatáscsökkenést eredményezett mind a receptor kötési, mind pedig a G-protein aktivációs tesztekben. Kísérleteink azt támasztják alá, hogy az endomorfin-1 biológiai hatásait az amidált tetrapeptid molekula egésze hordozza, a szerkezet bármilyen csonkolása drasztikusan csökkenti a molekula receptorához való kötődését, ezáltal a töredék peptidek nem alkalmasak a G-fehérjéken keresztül zajló jelátviteli folyamatok elindítására sem.

A szintetikusan előállított opioid peptidanalógokkal kapcsolatos munkánk másik részében a Tyr-Pro-Phe-Phe-NH_2 szekvenciájú endomorfin-2 tetrapeptid C terminálison módosított analógjait vizsgáltuk szerkezet-hatástani adatok nyerése céljából. A természetes endomorfin-2-ben található karboxamid csoport kémiai módosításainak legtöbbje jelentős μ-receptor affinitás-csökkenéssel járt együtt és ezen ligandanalógok nem növelték a membránpreparátum [^{35}S]GTPγS inkorporációját sem. A figyelemre érdemes kivétel az endomorfin-2-ol származék volt, amelyben a karboxamid funkciót alkoholos hidroxi-csoport helyettesítette. Az endomorfin-2-ol a szülővegyületnél nagyobb μ-receptor affinitással rendelkezett. Kimutattuk továbbá, hogy a C-terminális aromás gyűrűjének távolsága a peptidváz gericéhez képest számottevően befolyásolja a ligandanalógok receptor kötési és funkcionális biokémiai tulajdonságait. Noha a karboxamid csoport az affinitás jelentős csökkenése nélkül távolítható el a peptid C-terminálisáról, a hatékony receptor-közvetített G-fehérje stimulálás és a naloxonnal való gátolhatóság (az antagonizmus opioid jellege) szempontjából legalább egy poláros csoport jelenléte szükséges a C-terminálison. Hatástani vizsgálataink arra utalnak, hogy az endomorfin-2 amidált C-terminálisa alapvető szerepet játszik a peptid kötődésében és a ligandnak farmakológiai kísérletekből megismert agonista/antagonista tulajdonságaiban.

7. LIST OF PUBLICATIONS

1. Szatmari I., **Biyashev D.**, Tomboly Cs., Toth G., Szabo Gy., Macsai M., Borsodi A. and Lengyel I.. Influence of degradation on receptor binding properties and biological activity of Endomorphin 1. (2001) Biochem. Biophys. Res. Commun., 284(3):771-6.
2. **Biyashev D.**, Monory K., Benyhe S., Schutz J., Koch M., Schmidhammer H. and Borsodi A. Novel delta opioid receptor selective ligands in the 14-alkoxy-substituted indolo- and benzofuromorphinan series. (2001) Helvetica Chimica Acta, 284, 2015-2021.
3. Lengyel I., Orosz G., **Biyashev D.**, Kocsis L., Al-Khrasani M., Rónai A., Fürst Zs., Tóth G. and Borsodi A.. C-Terminal modification and Phe^3-methylation changes the binding and agonist properties of endomorphin 2. (Accepted for publication in Biochem. Biophys. Res. Commun.).
4. **Biyashev D.**, Garadnay S., Marton J., Makleit S., Borsodi A. and Benyhe S. Biochemical characterization of newly developed β-etorphine and β-dihydroetorphine derivatives. (Submitted for Eur. J. Pharmacol.).

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