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The opioid properties of endomorphins in isolated organs and rat brain slices

Ph. D. dissertation

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SUMMARY

The opioid properties of endomorphins in isolated organs and rat brain slices

My studies were aimed at the characterization of opioid properties of recently discovered brain peptides, endomorphins (Tyr-Pro-Trp-Phe-NH_2, endomorphin-1, EM-1 and Tyr-Pro-Phe-Phe-NH_2, endomorphin-2, EM-2) and their synthetic analogs, using different in vitro pharmacological techniques. Since natural endomorphins have been reported to possess potent and μ-opioid receptor type-selective agonist effect and, surprisingly, also partial agonist properties, special attention was paid to these issues. The μ-opioid receptor-selective agonist enkephalin analog (DAMGO), morphiceptin, morphine and normorphine were used as reference agonists. Technically two types of in vitro systems were used: μ-opioid receptor-containing, field-stimulated i) isolated organs (mouse vas deferens, MVD hosting d, μ and ? opioid receptors and guinea-pig ileum, GPI, μ, ?) and ii) rat brain slices (nucleus tractus solitarii-dorsal motor vagal nucleus complex, NTS-DVN). The endomorphin analogs were modified in position 1, 2 and 4 as compared to the parent natural peptides. 3' ring hydroxylation on Tyr^1 with or without a-methylation resulted in a loss in agonist potency whereas 2', 6'-dimethylation (Dmt) increased potency considerably as assayed in MVD. Substitution of Pro^2 by D-Met, D-Ser or cycloSer but not by L-Ser or Hyp yielded analogs with potencies comparable to that of parent peptide. Substitution of D- or L-Ser in position 4 in the D- or L-Ser^2-substituted analogs caused further loss in agonist potency. Free carboxylic terminus reduces potency whereas the change of amide function for an alcoholic one (-ol-derivatives) preserves agonist activity. In addition the agonist actions were exerted at the μ-opioid receptor type both in MVD and GPI with the exception of the derivative with a free C-terminus. This latter tendency matches the one for morphiceptin and its free carboxylic pair. Using the partial μ-opioid receptor pool inactivation strategy by ß-funaltrexamine in MVD natural endomorphins, their -ol-derivatives and ?Dmt?^{1} -EM-1 were found partial agonists whereas ?D-Met?^{2}-EM-2 is a possible full agonist. DAMGO, DAMGA and morphiceptin were full agonists, normorphine was a possible full agonist whereas morphine was a partial μ-opioid receptor agonist. In adult rat NTS-DVN slices the a_2-adrenoceptor agonist clonidine, DAMGO and both natural endomorphins inhibited the field stimulation-induced release of ^{3}H-norepinephrine (^{3}H-NE). However, DAMGO had shown dose dependent inhibitory effect but endomorphins did not even in the presence of dipeptidyl-aminopeptidase IV inhibitor, Diprotin A. One of the possible explanations of this phenomenon is that endomorphins behave as partial agonists also in the NTS-DVN complex.

ÖSSZEFOGLALÁS

Az endomorfinok opioid tulajdonságai izolált szervekben és patkány agyszeleteken

Tanulmányom célja az, hogy jellemezze a nemrég fölfedezett agyi peptidek, az endomorfinok, (Tyr-Pro-Trp-Phe-NH_2, endomorfin-1, EM-1 and Tyr-Pro-Phe-Phe-NH_2, endomorfin-2, EM-2) és ezek szintetikus analógjainak tulajdonságait különbözo in vitro farmakológiai technikákkal. A természetes endomorfinokról kimutatták, hogy eros és szelektív µ-opioid agonisták, de meglepo módon, parcialis agonista tulajdonságokkal is rendelkeznek. Ezért érdemel különös figyelmet ez a téma. µ-Opioid receptor szelektív agonistákat használtunk referensként: az enkefalin származék DAMGO-t, a morficeptint, a morfint és a normorfint. Két in vitro technikát alkalmaztunk: a.) izolált szerveket – egér vas deferenst (MVD), mely d, µ és ?, valamint tengerimalac ileumot (GPI), mely µ és ? opioid receptorokat tartalmaz–, és b.) patkány agyszeleteket – melyben nucleus tractus solitarii-dorsal motor vagal nucleus complex (NTS-DVN) található.

Az eredeti endomorfinokat illetve azoknak az 1, 2 és 4 helyzetben módosított analógjait használtuk. Az elso két analóg, ahol a Tyr^1 gyuru 3' helyén csak hidroxilcsoport van illetve ezen kívül még egy a-helyzetu metilcsoport is található gyenge agonistának bizonyult ugyanakkor a 2', 6'-dimetil szubsztitució (Dmt) eros agonista hatást eredményezett az MVD-ben. Ha a Pro^2-t helyettesítjük D-Met-el, D-Ser-el vagy cycloSer-el, de nem L-Ser-nel vagy Hyp-al akkor az így kapott analógok hatáserossége az eredeti peptidéhez hasonló. Ha a D- vagy L-Ser^2 analógoknál a 4 helyzetben D- vagy L-Ser-t szubsztituálunk, akkor tovább csökken az agonista hatás erossége. A C-terminális helyen a szabad karboxilcsoport csökkenti, viszont az alkohol- vagy amidcsoport megorzi az agonista aktivitását. Az általunk használt vegyületek, a C-terminális szabad karboxilcsoportot tartalmazók kivételével, az MVD-ben és GPI-ben lévo µ-receptorokon keresztül fejtették ki hatásukat. Hasonló tendencia figyelheto meg a morficeptinnél és annak C-terminális karboxilcsoportot tartalmazó analógjánál is. A részleges µ-opioid receptor inaktivációs stratégiához a ß-funaltrexamint alkalmaztuk MVD-en és azt találtuk, hogy endomorfinok és azok alkohol származékai, a ?Dmt^1?-EM-1, részleges agonisták, míg statisztikailag lehetséges, hogy a ?D-Met^2?-EM-2 teljes agonista. A DAMGO, DAMGA és a morficeptin teljes agonisták, a normorfin lehetséges, hogy teljes agonista, míg a morfin csak részleges µ-opioid receptor agonista. Az a_2-adrenoceptor agonista (Klonidin), DAMGO és az endomorfinok gátolták a ^{3}H-noradrenalin (^{3}H-NA)-nak az elektromos téringerlés hatására történo felszabadulását a felnott patkányok vagus komplexének (NTS-DVN) szeletkészítményén. Míg a DAMGO hatása dózisfüggo, az endomorfinok hatásai nem, még a dipeptidyl-aminopeptidaz IV gátló diprotin A jelenlétében sem. A jelenség egyik lehetséges magyarázata az, hogy az endomorfinok részleges agonistákként viselkednek az NTS-DVN komplexben is.

ABBREVIATIONS

1/?A?	**reciprocal of equieffective agonist concentration in the absence of the partial receptor inactivator.**
1/?A'?	**reciprocal of equieffective agonist concentration after the partial receptor inactivation.**
?A?	**agonist concentration.**
?A'?	**equieffective agonist concentration in the presence of partial receptor inactivator.**
AUC	**area under the curve.**
?? MeDopa^{1}?-EM-2	**?? Me,3'OH L-Tyr^{1}?-endomorphin-2.**
? -FNA	**beta-funaltrexamine.**
?B?	**antagonist concentration.**
Ci	**curie.**
CNS	**central nervous system.**
?cycloSer^{2}?-EM-2	**?cycloSerine^{2}?-endomorphin-2.**
DAMGA	**?D-Ala^{2}, NMePhe^{4}, Gly^{5}-NH$_2$?-enkephalin.**
DAMGO	**?D-Ala^{2}, NMePhe^{4}, Gly^{5}-ol?-enkephalin.**
DAP-IV	**dipeptidyl-aminopeptidase IV(DPIV, EC 3.4.14.5).**
Diprotin A	**IPI (Ile-Pro-Ile).**
Dmt^{1}-EM-1	**?2', 6'-Dimethyl-L-tyrosine^{1}?-endomorphin-1.**
Dmt^{1}-EM-2	**?2', 6'-Dimethyl-L-tyrosine^{1}?-endomorphin-2.**
DR	**dose ratio.**
DT-II	**deltorphin-2.**

?D-Met^2?-EM-2	**?D-Met^2?-endomorphin-2.**
?Dopa^1?-EM-2	**?3'OH L-Tyr^1?-endomorphin-2.**
?D-Ser^2?-EM-2	**?D-Ser^2?-endomorphin-2.**
?D-Ser^2, D-Ser^4?-EM-2	**?D-Ser^2, D-Ser^4?-endomorphin-2.**
EM-1	**endomorphin-1.**
EM-1-ol	**endomorphin-1-ol.**
EM-2	**endomorphin-2.**
EM-2-ol	**endomorphin-2-ol.**
GPI	**Longitudinal muscle strip-Auerbach plexus of guinea-pig ileum.**
^{3}H-NE	**Levo-?ring-2,5,6-^{3}H?-norepinephrine.**
?Hyp^2?-EM-2	**?Hydroxyprolyl^2?-endomorphin-2.**
IC_{50}	**50% inhibitory concentration.**
K_A	**dissociation constant for agonist.**
K_e	**equilibrium dissociation constant for antagonist.**
?L-Leu^4-OH?-EM-2	**?L-Leu^4-OH?-endomorphin-2.**
?L-Phe^4-OH?-EM-2	**?L-Phe^4-OH?-endomorphin-2.**
?L-Pro^4-OH?-Mor	**?L-Pro^4-OH?-morphiceptin.**
?L-Ser^2?-EM-2	**?L-Ser^2?-endomorphin-2.**
?L-Ser^2, L-Ser^4?-EM-2	**?L-Ser^2, L-Ser^4?-endomorphin-2.**
MVD	**mouse vas deferens.**
NTS	**the nucleus of the solitary tract.**
NTS-DVN	**nucleus tractus solitarii-dorsal vagal nucleus.**
Ntx	**naltrexone.**

NX	**naloxone.**
OFQ	**orphanin FQ.**
ORL-l	**opioid receptor-like receptor 1.**
q	**fraction of receptors remaining after partial receptor inactivation.**
S	**stimulus, in the Stephenson occupation theory, it is proportional to the fractional receptor occupancy.**
S.E.M	**standard error of mean.**
S_1	**AUC above the baseline after the first electrical field stimulation.**
S_2	**AUC above the baseline after the second electrical field stimulation.**

INTRODUCTION-OVERVIEW OF THE LITERATURE

Naturally occurring opioid peptides can produce their effects through their interaction with one or more type of opioid receptors (?, ?, ?) regardless of their sources.

On the basis of amino acid sequences at the N-terminal, two types of mammalian endogenous opioid peptides exist, one containing Tyr-Gly-Gly-Phe as the message domain (enkephalins, endorphins and dynorphins) and the other containing the Tyr-Pro-Trp/Phe sequence (endomorphin-1 and -2).

The first endogenous agonists for opioid receptors, enkephalins (?Met?enkephalin, Try-Gly-Gly-Phe-Met and ?Leu?enkepalin, Try-Gly-Gly-Phe-Leu) were isolated and identified from mammalian brain by Hughes and his coworkers in 1975 ?63?. At a short notice enkephalins were proposed as the endogenous ligands for opiate receptor sites because of their agonist activity both in MVD and in GPI. However, the differences between the activities of morphine and enkephalins in in vitro bioassays namely, MVD and GPI led to the discovery of the ?-opioid receptor ?86?. Moreover both peptides were found to display higher affinity to ? than to ?-opioid receptors. The second endogenous opioid peptide, ?-endorphin was discovered in 1976 ?84? and found to be potent at both ? and ?-opioid receptors ?1?. Dynorphins were discovered by Goldstein and his coworkers in 1979 ?45?. They represent the last of the three currently known families of endogenous opioid peptides. In contrast to enkephalins and ?-endorphin, dynorphins preferentially bind to ?-opioid receptors ?23?. Recently two peptides, endomorphin-1 and endomorphin-2 have been isolated from bovine ?181? then from human brain ?50? and they had shown high affinity and selectivity toward ?-opioid receptors. They were proposed to be the fourth family of mammalian endegenous opioid peptides.

In 1995 endogenous opioid-like peptide named nociceptin by one group ?99? and orphanin FQ (OFQ) by other ?133? was isolated and had shown a significant sequence homology to dynorphin A with the similar length of 17 amino acids, identical amino acids residues at C-terminal and slight modification at the N-terminus (Phe-Gly-Gly-Phe instead of Tyr-Gly-Gly-Phe). The presence of phenylalanine in the N-terminal is sufficient to abolish interaction of this peptide with the three classical opioid-peptide

receptors. Nociceptin interacts with so called opioid receptor like (ORL1) receptor which was accepted as a member of the family of opioid receptors on the basis of its structural homology towards the classical opioid receptor types though the discrepancy in the pharmacological effect is still present ?108?. In addition the inhibitory effect of this peptide on electrically stimulated contractions of MVD and GPI was insensitive to the opioid receptor antagonist naloxone ?182?.

Naturally occurring opioid peptides (dermorphin, dermenkephalin and deltorphins) have been isolated from amphibian skin. They have an amino acid sequence of Tyr- (D-Ala/D-Met)-Phe at their N-terminus. These are the non mammalian amphibian skin peptides dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and dermenkephalin (Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2) which was also named as deltorphin ?35? because of its high affinity and selectivity for ?-opioid receptors. They were extracted from the skin of the Argentinean frog Phyllomedusa sauvagei by Montecucchi ?106? and Kreil and their coworkers ?75? respectively. Dermorphin is a ?-selective agonist without significant affinity at ?- and ?-opioid receptors ?4; 13; 140?. The first highly selective, potent, natural ?-opioid receptor agonist peptides were named as ?D-Ala^2?-deltorphin-I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) and ?D-Ala^2?-deltorphin-II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) to differentiate them from deltorphin ?35?.

The selective interaction of dermorphin with ?-opioid receptors through its N-terminal Tyr-D-Ala-Phe-Gly sequence ?107? led to the synthesis of many potent analogs such as DALDA (Tyr-D-Arg-Phe-Lys-NH_2) and TAPS (Tyr-D-Arg-Phe-Sar, Sar= N-methylglycine) ?143; 145?.

Before the discovery of endomorphins naturally occurring opioid peptides with similar structures to that of endomorphins were found. They preferentially bound to ?-opioid receptors. These were ?-casomorphin (Tyr-Pro-Phe-Pro-Gly), hemorphin (Tyr-Pro-Trp-Thr), Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) and Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2). Their structures were based also on Tyr-Pro-X (where X is Phe, Trp or aliphatic amino acid) sequence in their N-terminal. ?-casomorphin ?54? and hemorphin ?12? were isolated from heptapeptide fragment of bovine beta-casein (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) and from digests of hemoglobin respectively. Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2,

MIF= melanocyte-stimulating hormone release inhibiting factor= Pro-Leu-Gly-NH_2) and Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2) were isolated from bovine hypothalamus and human brain cortex [33; 49; 58; 59]. Interestingly Tyr-MIF-1 and Tyr-W-MIF-1 were found to act as agonists as well as antagonists at δ-opioid receptors [34; 180]. δ-Casomorphin-derived opioid tetrapeptide morphiceptin (Tyr-Pro-Phe-Pro-NH_2) was the first representative of Tyr-Pro group displaying morphine like activities and selectivity for μ-opioid receptors [22]. It should be recalled that in the enkephalin-endorphin-dynorphin-related group of endogenous opioid peptides even a moderate preference for the κ-opioid receptor type was rather exceptional whereas a selectivities/preferences for the δ or μ type were obviously present (for review see [62].) In addition no endogenous ligand has been discovered in mammals with preferential binding to κ-opioid receptor until 1997, though the plant- derived alkaloid, morphine and related compounds which are clinically very important in relieving pain were reported to act primarily through μ-opioid receptors [95]. However, on the one hand morphiceptin was effective only in the higher nanomolar or low micromolar concentration range and on the other hand no morphiceptin-related peptide could be detected in mammalian brain.

The real breakthrough came in 1997 with the discovery of endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2; EM-1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2; EM-2), the first mammalian brain opioid peptides having high agonist potency and selectivity for μ-opioid receptor [50; 181]. These endogenous opioid tetra peptides were not only highly potent and selective μ-opioid receptor agonists [181] but their N-terminal amino acid sequence differed from those of previously known endogenous mammalian opioid peptides. Another distinctive feature is that while the precursors pro-opiomelanocortin (the precursor of β-endorphin), pro-enkephalin (the precursor of [Met^5]- and ([Leu^5]enkephalin), pro-dynorphin (the precursor of dynorphins) [66; 109; 116] are well known no such a precursor for endomorphin-1 and -2 has hitherto been detected. Furthermore some other items are also missing to consider these peptides as neurotransmitters, neurohormones or hormones, such as the solid proof of the Ca^{2+} dependent release [60].

Results obtained by use immunocytochemical and in situ hybridization demonstrate that enkephalins and dynorphins are widely distributed throughout the CNS including spinal trigeminal nucleus, the spinal cord dorsal gray laminae (in which terminate the first-order somatosensory neurons carrying signals including of nociceptive type) as well as NTS (nucleus tractus solitarii) (where viscerosensory signals of autonomic character relayed or terminate) whereas the existence of ß-endorphin is relatively limited (mainly in the arcuate nucleous and NTS ?10; 68; 83; 147; 178?. Enkephalins, ß-endorphin, dynorphins were also detected in the peripheral tissues including the vas deferens ?71; 152? as well as in the enteric nervous system of guinea pigs ?30; 31; 79?. Recent studies reveal that endomorphin-1 widely distributed throughout the brain ?94? whereas endomorphin-2 is more prevalent in the spinal cord in particular in superficial laminae of spinal dorsal horn and in nucleus of spinal trigeminal tract ?93; 174?.

The concept of the multiple receptors was proposed first by Martin and his coworkers ?92?. After two decades of hard work carried out in many laboratories, now it is clear that there are three well defined or classical types of opioid receptors namely, ?, ? and ?. Cloning of these receptors ?25; 36; 40; 69; 85; 97; 102; 179? have led to farther understanding the function of opioid receptors at the molecular level. In addition, the sequence analysis of these cloned receptors revealed that opioid receptors belong to the superfamily of G-protein-coupled receptors. Moreover these receptors are highly homologous in their protein structure ?70?. Opioid receptors are coupled through G-proteins of the pertussis toxin-sensitive Gi/Go family ?78?. The effectors include adenylyl cyclase ?25; 36; 40; 69; 85; 97; 102; 179?, N- and L-type Ca^{2+} channels ?124; 125; 168?, phospholipase C ?65; 158? and inwardly rectifying K^{+} channels ?53?. Opioid receptor activation have been reported to inhibit adenylyl cylase ?149? and Ca^{2+} channels ?55; 162? and activate K^{+} channels ?117?, though increase of intracellular Ca^{2+} level has also been reported ?64?. Both the limitation of Ca^{2+} entry and the hyperpolarization of the cells may give tenable explanation for the opioid blockade of transmitter release.

At early stage, the G-protein activation can be measured by the binding of the nonhydrolyzable GTP analog, guanosine-5'-O-(3-?^{35}S?thio) triphosphate (?^{35}S?GTP?S)

to isolated membranes in the presence of excess guanosine diphosphate (GDP) [57; 87; 153; 171]. At cellular and tissue level the agonist efficacy can be measured by the ability of agonists to induce maximal [^{35}S]GTPγS binding to the cell or tissue membranes upon their interaction with G-protein coupled receptors [32; 88; 148; 171].

Endomorphins stimulate the binding of ^{35}S-GTP-γ-S to the membranes of μ-opioid receptor-containing cells or tissues [3; 51; 105; 154]. Endomorphins inhibit calcium channels [56; 101] following their interaction with μ-opioid receptors. This inhibition of Ca^{2+} current in periaqueductal grey (PAG) failed to take place in μ-opioid receptor-deficient mice [26]. It has been shown that endomorphins activate inwardly rectifying potassium channels of Xenopus oocytes co-expressing μ-opioid receptors with G-protein-activated K^+ channels [46]. These effects of endomorphins were found to be surmounted by μ-opioid receptor antagonists [3; 67; 104; 110; 111; 159], but not of δ- or κ-antagonists [67; 110]. Endomorphins inhibit electrically induced muscle contractions in GPI preparations [96; 139; 170; 181] by depressing acetylcholine release from the myenteric plexus [115], similarly to DAMGO [74], another μ-opioid receptor selective agonist [115]. Natural endomorphins have shown to produce spinal and supraspinal, μ-opioid receptor-mediated analgesia [44; 139; 157; 161; 181]. Systemic administration of endomorphins has been reported to cause vasodilatation in rat [16; 17; 27; 28], rabbit [18] and mice [19]. However, there is an apparent controversy about the mechanism of this property [19; 20; 134].

One of the major tasks of medicinal chemistry is to produce therapeutically useful drugs by proper, inventious modification of the structure of biologically active natural substances. To do this first the pharmacodynamic properties of natural substances should be characterized to find leads as to the tests relevant to the desired as well as the unwanted actions of the substance. Then by suitable synthetic procedures novel structures should be produced and, in turn, by studying the biological properties of resulting substances the structure-activity relationships must be established to set up a design rationale for further development.

One of my tasks was to characterize the opioid effects of novel endomorphin congeners where the synthetic modifications, in part, followed the strategies which were successful in other opioid peptides.

For the general characterization of opioid properties of natural endomorphins and their novel analogs, we used primarily the mouse vas deferens (MVD) bioassay. We chose this tissue for this purpose because it is the isolated organ that contains all three major opioid receptor types i.e. ?, ? and ? ?82; 86?. However, for prominently interesting novel analogs besides MVD we used another bioassay, the longitudinal muscle stripe of guinea-pig ileum (GPI) which contains opioid receptors only of the ?- and ?-types ?82; 86; 175?.

Some reports indicated that endomorphins acted as partial agonists in the ?^{35}S?GTP?S-binding assay ?3; 61; 104; 112; 154?, which is unusual feature in a first messenger. We decided to investigate this issue by using a pharmacological approach. To obtain receptor constants for the agonists ?42? in the mouse vas deferens, the strategy of partial, irreversible inactivation of ?-opioid receptors by the alkylating analog of naltrexone, ?-funaltrexamine ?129? was used. ?-funaltrexamine has been characterized mainly as an irreversible ?-opioid receptor antagonist and reversible ?-opioid receptor agonists ?39; 52; 163; 176; 177?. These receptor constants enabled us to assess the partial properties of these peptides and also of nonpeptides.

The NTS-DVN (nucleus tractus solitarii-dorsal vagal nucleus) is a complex circuitry of local interneurons and projection neurons signaling via biogenic amine, amino acid and peptide neuroregulators and their respective receptors ?120?. It has been reported that opioids in the NTS influence cardiovascular effects and catecholamine release ?6; 29?. In addition, NTS contains abundant stores of endogenous catecholamines (A2 and C2 cell groups). The NTS-DVN has a prominent ?-opioid receptor density among the brainstem areas ?90?. Endomorphins have a characteristic distribution pattern in the NTS-DVN ?94?. Furthermore the ?-opioid receptor selective agonist enkephalin analog DAMGO has been reported to inhibit stimulation-induced ^{3}H-norepinephrine (^{3}H-NE) release in rat NTS-DVN slices ?7?. These finding indicated the modulatory effect of μ-opioid receptors in norepinephrine release from NTS in vivo as well as in vitro. For these reasons we extended our interest to analyze the effect of endomorphins on the electrically stimulated release of ^{3}H-norepinephrine from NTS-DVN slices of rat in vitro.

AIM OF THE STUDY

The present study was designed to:

1.) Explore the structure-activity relationships of novel endomorphins analogs in isolated organs.

2.) Determine the affinities and efficacies of endomorphins, ?2',6'-Dimethyl-L-tyrosine[1]?- endomorphin-1, endomorphin-1- and –2-ol, ?D-Ser[2]? and ?D-Met[2]?-endomorphin-2, morphiceptin, ?D-Ala[2], NMePhe[4], Gly[5]-ol?-enkephalin (DAMGO), ?D-Ala[2], NMePhe[4], Gly[5]-NH_2?-enkephalin (DAMGA) as well as morphine and normorphine as non peptide μ-opioid agonists in mouse vas deferens.

3.) Measure the inhibitory effect of endomorphin-1 and endomorphin-2 on field electrical stimulus induced ^{3}H-norepinephrine release from rat NTS-DVN slices.

MATERIALS AND METHODS

Drugs

Endomorphin-1, endomorphin-2, endomorphin-1-ol, endomorphin-2-ol, ?L-Leu^4-OH?-endomorphin-2, ?L-Phe^4-OH?-endomorphin-2, ?D-Ser^2?-endomorphin-2, ?D-Met^2?-endomorphin-2, ?L-Ser^2?-endomorphin-2, ?D-Ser^2, D-Ser^4?-endomorphin-2, ?L-Ser^2, L-Ser^4?-endomorphin-2, ?cycloSer^2?-endomorphin-2, ?Hydroxyprolyl^2?-endomorphin-2, ?? MeDopa^1?-endomorphin-2, ?Dopa^1?-endomorphin-2, morphiceptin, ?L-Pro^4-OH?-morphiceptin, ?D-Ala^2, NMePhe^4, Gly^5-ol?-enkephalin (DAMGO), ?D-Ala^2, NMePhe^4, Gly^5-NH_2?-enkephalin (DAMGA) and diprotin A were synthetised by the Research Group of Peptide Chemistry of Hungarian Academy of Sciences at Eötvös University Budapest. The details of the synthesis have been given elsewhere ?2; 11; 72; 119; 139?.

Deltorphin-II was kindly supplied by G. Tóth (Biological Research Center of Hungarian Academy of Sciences, Szeged) as well as ?2',6'-Dimethyl-L-tyrosine^1?-endomorphin-1 and ?2',6'-Dimethyl-L-tyrosine^1?- endomorphin-2.

Naltrexone hydrochloride and naloxone hydrochloride were a gifts from Du Pont Pharmaceuticals, Geneva, Switzerland. ?-funaltrexamine hydrochloride was purchased from Tocris Cookson Ltd. (Bristol, UK). Morphine sulfate and normorphine base were obtained from ICN Alkaloida Ltd. (Tiszavasvári, Hungary). ^{3}H-norepinephrine (2.1 TBq= 56.3 Ci/mmol) was purchased from NEN (Boston, USA). Clonidine hydrochloride was obtained from Sigma-Aldrich (St. Louis, USA), ascorbic acid from Reanal (Budapest, Hungary). All the other substances used were of analytical grade and purchased either from the Sigma-Aldrich Co (St. Louis, USA) or Reanal (Hungary).

Drugs were dissolved in bidistilled water and in some cases with the addition of drops of 0.1n HCl or ethanol. Krebs solution was used for subsequent dilutions.

Animals

Male CFLP mice, non-albino guinea-pigs and Wistar/ Wistar rats were used through out the experiments. Animals were purchased from LATI or Charles River, Hungary. They were kept in groups in temperature- and humidity-controled room at a 12-h light/ dark cycle. The conditions of animal housing and experimentation followed

ethical guidelines set by the Ethical Board of Semmelweis University, based on EC Directive 86/609/EEC.

Every efforts were made to minimize the number and any suffering of animals used in the experiments from which the data of this thesis were obtained.

Methods

I. Isolated organs

A.) Mouse vas deferens. Vasa deferentia removed from CFLP (Carworth Europe Farm, Lanne-Patter, ICI Alderly Park I. stock) mice weighing 35-45 g were prepared and used as previously described ?135?. Briefly, after decapitation, the abdominal viscera were exposed with a mid-line incision and the vasa deferentia were rapidly removed from the animals. The organs (a single vasa/bath) were mounted under an initial tension of 0.1 g in Mg^{2+}-free Krebs' solution of the following compisition (mM/L): NaCl, 118.0; $NaHCO_3$, 25.0; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 2.5; glucose, 11.0 aerated with carbogen (O_2: CO_2= 95: 5) at 31^{o}C. Field electrical stimulation (upper ring, lower straight wire electrode arrangement) was used. The parameters of stimulation were as follows: pairs (100 ms pulse distance) of rectangular impulses (1 ms pulse width, 9 V/cm i.e. supramaximal intensity) were repeated by 10s. The contractions were monitored by a.transducer connected to amplifier and recorder.

B.) Longitudinal muscle strip/Auerbach plexus of guinea-pig ileum. Male, non-albino guinea-pigs weighing 400-500g were used. The strips were prapared according to Paton and Vizi ?121? and the experimental conditions were the same as used previously ?135?. In brief, the animals were killed by decapitation and a segment of ileum not including the 10 cm nearest the ileo-cecal junction was taken. The longitudinal muscles were carefully removed from circular muscles by cotton balls wetted in Krebs. 25-40 mm long muscle strips were mounted under an initial tension of 0.8g in Krebs' solution of the following composition (mM/L): NaCl, 118.0; $NaHCO_3$, 25.0; KCl, 4.7; KH_2PO_4, 1.2; glucose, 11.0; $CaCl_2$, 2.5; $MgSO_4$, 1.2 aerated with carbogen at 36?C. The parameters of field stimulation were as follows: supramaximal (1 ms pulse width, 9V/cm intensity) rectangular impulses delivered at 0.1 Hz frequency.

The muscle contractions were monitored by a.transducer connected to amplifier and recorder.

Experimental paradigms

Mouse vas deferens and longitudinal muscle strip/Auerbach plexus of guinea-pig ileum

In the isolated organ series, 30-40 min equilibration was used for mouse vas deferens, 45-60 min for guinea-pig ileum under stimulation. The dose-response curves for the agonists were constructed in non-cumulative manner; the drug exposure was less than 2 min with the exception of Dmt^1-endomorphins, where it was 10-25 min. Then tissues were washed and allowed to regain their pre-drug twitch height before subsequent durg administration. The administration cycle was 12-18 min, with 3-5 interim washes with the exception of Dmt^1-endomorphins, where it was 40-60 min with 8-12 washes. The preparations were equilibrated with naltrexone for 20 min; the single-dose method ?73? was used for assessing the antagonism in guinea-pig ileum, whereas complete dose-response curves were taken with the agonists also in the presence of antagonists in mouse vas deferens. Based on the results of pilot experiments, a 30 min exposure time to $5x10^{-7}$ M ?-funaltrexamine was chosen to carry out experiments for determining the receptor constans of opioid agonists in mouse vas deferens. In the control period, always in paired arrangement, endomorphin-1, endomorphin-1-ol, DAMGA and DAMGO dose response curves were constructed from four to sex pre-set concentrations, followed by a single concentration of deltorphin-II. The incubation conditions with ?-funaltrexamine were the same when we extended the analyses to ?2', 6'-dimethyl-L-tyrosine^1?-endomorphin-1, endomorphin-2, endomorphin-2-ol, ?D-Ser^2?-endomorphin-2, ?D-Met^2?-endomorphin-2, morphiceptin, morphine and normorphine with the exception that we did not give a single dose of deltorphin-II. In one paired experiment, a crossover design was used, i.e. the same preparation received alternately endomorphin-1 and the -ol derivative; these results were not pooled with the others. Exposure to ?-funaltrexamine was followed by a 40-60 min washout period with 8-12 washes. A present criterion for inclusion, which has been recommended by Ward and his coworkers ?176?, was at least 80% recovery. After recovery a selected dose of

agonists was repeated until the responses became stabilized; it took 2-4 repetitions. Thereafter the agonist effects were tested at four-six further, pre-set concentration levels. At the end of administration cycle, deltorphin-II was added in the series of experiment planed for the determination of receptor constants for endomorphin-1, endomorphin-1-ol, DAMGA and DAMGO. In the series of experiments design for determination the receptor constants of ?2', 6'-Dimethyl-L-tyrosine[1]?-endomorphin-1, endomorphin-2 and 2-ol, ?D-Ser[2]?-endomorphin-2, ?D-Met[2]?-endomorphin-2, morphiceptin, morphine and normorphine the same circumences were kept with exception we carried out the experiments without deltorphin-II addition.

Evaluation

A.) Agonist activity in mouse vas deferens and guinea-pig ileum.

In isolated organs, the IC_{5O} values were calculated from the logarithmic regressions of individual dose-response curves. For determining the parameters of antagonism (dose ratio, DR and equilibrium dissociation constant, K_e) by the competitive opioid receptor antagonist naltrexone, either the single-dose method (in guinea-pig ileum, ?73?) was used or they were calculated from complete dose-response curves taken in the absence and presence of antagonist (in mouse vas deferens, ?8?). Both in MVD and GPI K_e values were caculated as:

K_e = ?B?/DR-1, where ?B? is naltrexone concentration. For the pooled IC_{50} and K_e values, the geometric means and the 95% confidence intervals ?38? were calculated.

B.) Agonist affinity in mouse vas deferens.

1.) Theoretical Part (quoted from Furchgott and Bursztyn 1967)

The theoretical basis for the procedure for obtaining dissociation constants of receptors-agonist complexes has been previously discussed in detail ?41?, but it is appropriate to consider briefly here the formulations used and the assumptions inherent in the theory.

The following relationship (modified from Stephenson, ?160?) is assumed to apply for steady-state conditions:

$$E_A/E_m = f(S) = f(?RA?) = f(?R_t?A?/K_A + ?A?) \quad (\mathbf{1}),$$

where E_A is the measured response and E_m is the potential maximal response of the responding tissue or effector; E_A/E_m is some function of the stimulus, S, and approaches 1 as S becomes very high; S equals the product of intrinsic efficacy, ?, times the concentration of receptor-agonist complex, ?RA?, of the effector; and ?R_t? is the total concentration of active receptors; ?A? is the concentration of free agonist in the region of and in equilibrium with the receptors, and K_A is the dissociation constant of RA. The equality of ?RA? with ?R_t??A?/ (K_A+?A?) comes from the low of mass action. It should be noted that ??R_t? is equivalent to Stephenson's efficacy term, *e*.

After irreversible inactivation of a fraction of the receptors, leaving a fraction, q, still active, one has

$E_{A'}/E_{m'}$=f (S') =f (??RA'?)= f??q?R_t??A'?/ K_A+?A'?? (**2**),

where $E_{A'}$, S', ?RA'? and ?A'? are equivalent to E_A, S, ?RA? and ?A? respectively, following reduction of ?R_t? to q?R_t?. When $E_{A'}$ equals E_A, then S' equals S, and ?RA'? equals ?RA?, and from the equations **1** and **2** it follows that:

1/?A?= (1-q) /q K_A +1/q*1/?A'? (**3**).

A plot of 1/?A? against 1/?A'? (reciprocals of equiactive concentrations of the agonist before and after inactivation) should fall on a straight line.

The value of the q should be equal to 1/(slope), and that of K_A should be (slope-1)/(intercept).

In developing the theory outlined above and in applying it to the analysis of actual experimental data, the following assumptions are made:

(a) That the agonist elicits a response of only one type in the effector and does so by reacting directly with only one type of receptor.

(b) That the population of this type of receptor is uniform with respect to K_A.

(c) That both before and after irreversible inactivation of a fraction of the receptors, E_A is the same function of S, and S has the same relationship to ?RA? (i.e., ? remains the same).

(d) That when E_A is measured, ?A? is essentially equal to the concentration of the agonist in solution bathing or perfusing the effector.

(e) That when E_A is measured, a steady state exists which is governed by the mass action law relating ?RA?/?R_t? to ?A? and K_A.

(f) That the agent used to inactivate the receptor irreversibly alters the sensitivity of the effector to the agonist only by reducing the concentration of active receptors for the agonist.

(g) That during the period over which the agonist is tested to obtain concentration-response data following washout of the inactivating agent, the fraction of receptors inactivated neither decreases nor increases significantly.

(h) That the formation of RA does not lead to inactivation (even transiently), so that when RA dissociates, the R released has the same potential for contributing to response as it had before complex formation (i.e., there is no desensitization due to reactions with A).

As will be apparent, precautions must often be taken in actual experiments to try to satisfy the conditions of the assumptions given above.

2.) Practical Part

In order to assess the receptor constants from the dose-response relationships obtained before and after ?-funaltrexamine exposure, sigma plot program was used for curve fitting to the respective set of points. For the „double reciprocal" plot (1/A versus 1/A' ?42; 164; 165? first the equiactive concentrations of agonist were taken at the suitable segment of dose-response curve before (A) and after (A') ?-funaltrexamine with 5 or 10% ordinate increments. For understanding see Fig. 1 panel A. Then the plot of 1/A against 1/A' (the reciprocals of equiactive concentrations of the agonist before and after the receptor irreversible partial inactivation) was performed. From the linear regression of double reciprocal plot, the active receptor fraction after ?-funaltrexamine (q) is given as 1/slope; for convenience, it was expressed in percent. The apparent dissociation constants of agonist (K_A) is given by (slope-1)/y intercept (Fig 1, panel B). The efficacy of drugs was calculated as the ratio of K_A over IC_{50}. For the presentation of pooled results geometric means and 95 % confidence intervals ?38? were calculated.

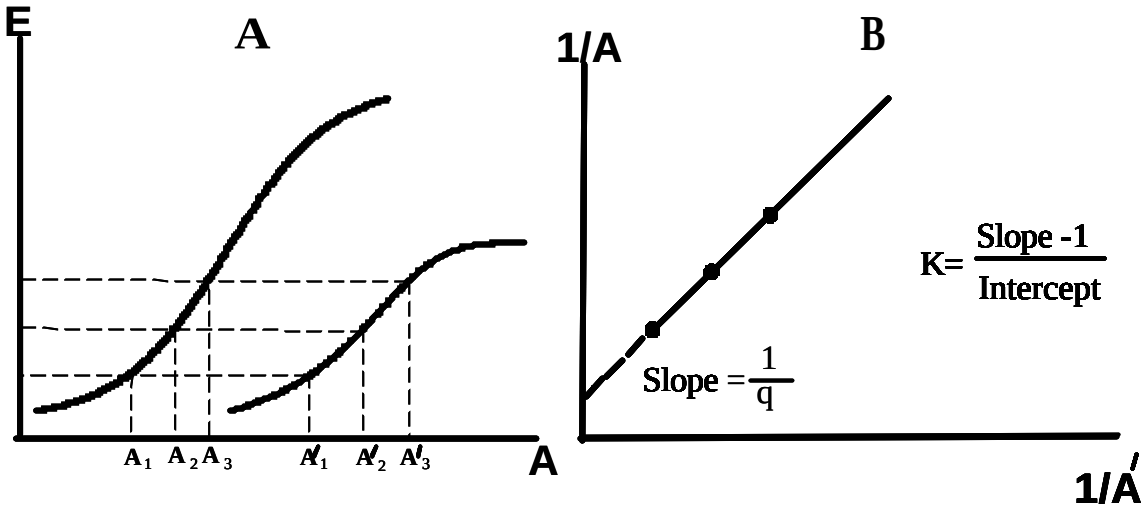


Fig. 1. The theoretical method of partial irreversible blockade for determining the agonist dissociation constant

II. NTS-DVN SLICES

Preparation, experimental paradigm

Male Wistar/Wistar rats weighing 180-260g were used. NTS-DVN slices were prepared and the experiments were carried out by a slightly modified version of the procedure described by Rónai et al.(in press), based on the method originally devised by Arakawa et al. ??. In brief, approximately 500 ? m thick coronal slice was cut below, at the level of and above the obex. From each slice a midline triangle (? 3.5 mm sides, apex down) was dissected and the apical parts were removed. The resulting trapezoids were divided in the midline and were cut into 200 ? m thick prisms with a MacIlwain chopper. The pooled prisms prepared from the two sides were passed to parallel loading and superfusion chambers, respectively. After equilibration for 30 min in 2.5 ml Krebs' solution containing 10^{-6} M ascorbic acid, aerated with carbogen at 36 ?C, the slices were loaded with 2.5-2.5 ? Ci ^{3}H-norepinephrine (56.3 Ci/mmol specific activity, NEN, Boston) for 15 min. The slices transferred to the superfusion chambers were superfused before collection with carbogen-saturated Krebs' containing no additives at 36 ?C for 50 min at a rate of 1.0 ml/min and for 10 min at 0.5 ml/min. Throughout the experiment

the rate of 0.5 ml/min was maintained with 3-min fractions; stimulation took place during fractions 7 and 17. The parameters were as follows: field stimulation, rectangular pulses of 2 ms width, 25 mA intensity, 2 Hz for 3 min. Drugs were added from fraction 12 with the exception of diprotin A which, in some experiments, was present in the superfusate from the last 10 min of pre-perfusion. At the end of experiments radioactivity remaining in the tissue was extracted with 1.0 ml 0.1 n HCl.

Evaluation

Release rate was expressed as percent of tissue content/fraction. Stimulation-induced release (S1 and S2) was characterized by the areas under the curve after stimulation, the arithmetic mean of last three samples before stimulation being taken as baseline. S_2/S_1 ratios were calculated and the drug actions were assessed by the alterations in S_2/S_1 ratios. The geometric mean and the 95% confidence intervals are given for the ratios ?38?. To characterize the effect of diprotin A preincubation (added 58 min before S1) on the stimulation-induced release, comparison by student's t-test for grouped samples was used.

STATISTICS

Both in isolated organs and brain slices analysis of variance (ANOVA) followed by Least Significant Differences (LSD) test ?156? for multiple comparisons was used for evaluation the significance of differences. All the results were transformed to logarithmic values before carrying out the statistical analysis. Student's t-test for grouped samples was applied. A probability of p? 0.05 was considered statistically significant. Throughout the thesis the molar concentrations of drugs were given (M or nM).

RESULTS

ANALYSIS OF OPIOID PROPERTIES IN ISOLATED ORGANS

A.) General opioid pharmacology of endomorphin-related peptides

Since one of the major aims of the study was to establish the opioid receptor type preference and agonist potency of a series of endomorphin-related, mostly novel peptide analogs, the following strategy was followed. First, the inhibitory concentration-response curves were constructed for the substances in isolated organs where ?-opioid receptors are present then the interaction between the ?-opioid receptor-preferring competitive antagonist naltrexone and the opioid agonist was determined. The paradigm whereby the interaction between an opioid agonist (here, DAMGO) and naltrexone is determined is demonstrated in Fig. 2 (upper panel, A). The inhibitory dose-response curves of agonist are constructed in the absence and in the presence of at least three different concentrations of antagonist. From the rightward shifts of dose-response curves by the antagonist (dose ratios, DR) a Schild-plot is constructed Fig. 2 (lower panel, B). A Schild slope not significantly different from unity (here from -1.00) denotes competitive type of antagonism; the intercept with the abscissa (pA2) is a characteristic measure of antagonist affinity. For a more accurate comparison, not the pA2 but the K_e values were calculated for the antagonists according to the equation given in the “method” section. The Schild plots for natural endomorphins are given in Fig. 3.

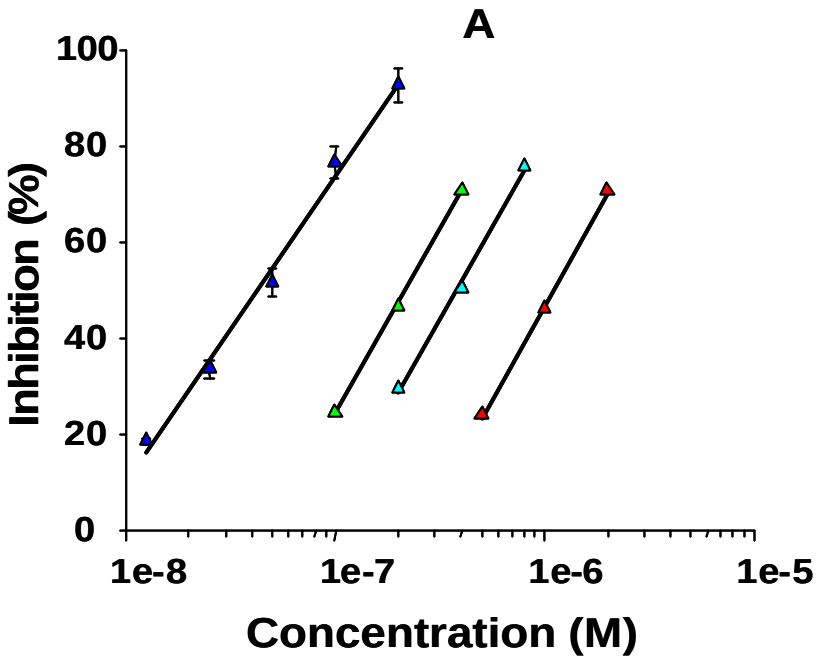


Fig. 2/A. The rightward shifts of DAMGO dose response curve in MVD caused by 1, 3 and 10 nM naltrexone (Ntx). Blue (control); green, cyan and red in the presence of 1, 3 and 10nM Ntx respectively (n=6)

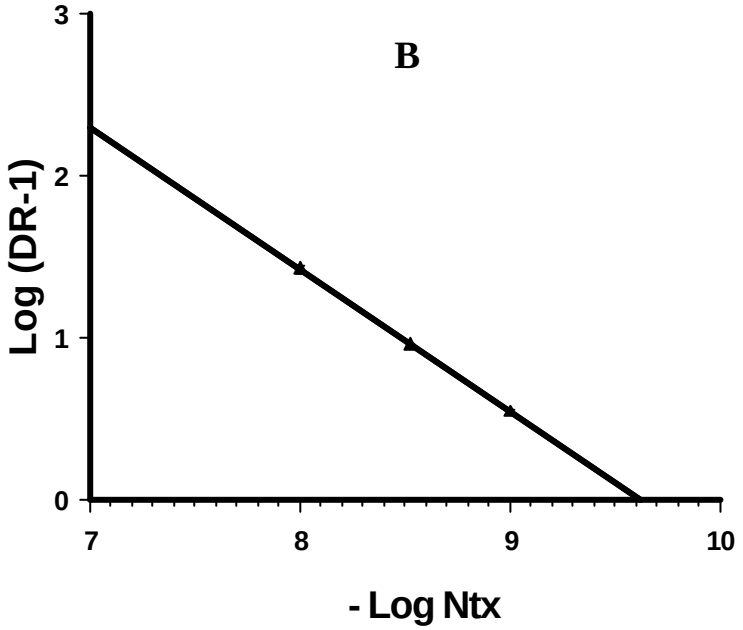


Fig. 2/B. Schild regressions in MVD, data points for naltrexone antagonism of responses to DAMGO. Ordinates: Logarithms of dose ratios-1. Abscissa: - logarithms of molar concentrations of naltrexone. Linear regressions were constructed from 6 animals.

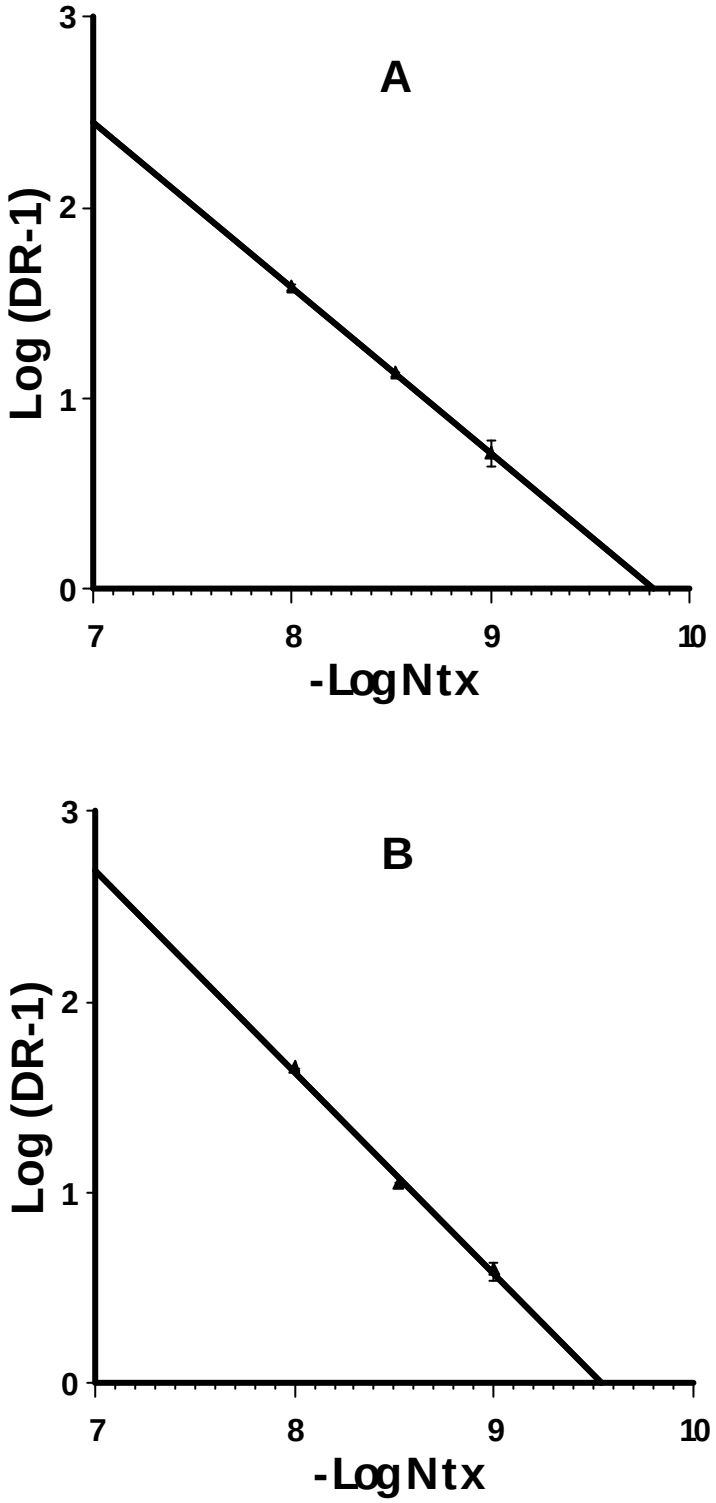


Fig. 3. Schild regressions in MVD, data points for naltrexone antagonism of responses to EM-1 (panel A) and EM-2 (panel B). Ordinates: logarithms of dose ratios-1. Abscissa: - logarithms of molar concentrations of naltrexone. Linear regressions were constructed from 6 animals for each compound.

In Table 1 and 4 it is shown how naltrexone antagonism can be taken as indicator of opioid receptor type preference. The naltrexone K_e in the range of 0.2-0.6 nM indicates agonist action at ?-opioid receptors whereas higher K_e values (characteristically, above 4) indicate agonism at other (in the case ?L-Phe^4-OH?-endomorphin-2 and ?D-Ala^2?Leu-enkephalin, delta) opioid receptor type. Intermediate K_e values, of course, reflect a mixed receptor type spectrum.

In the series of tetrapeptides having Tyr-Pro motif in their N-terminus the rank order of agonist activity in the mouse vas deferens was endomorphin-2 = endomorphin-1 ? endomorphin-2-ol ? endomorphin-1-ol ? Morphiceptin ? ?L-Phe^4-OH?endomorphin-2 ??L-Pro^4-OH?-morphiceptin ? ?L-Leu^4-OH?-endomorphin-2 (Table 1). A similar rank order of potency was obtained in the ginea-pig ileum (Table 1). The parent (amidated) endomorphin-1 and endomorphin-2 proved to be slightly potent than their -ol derivatives namely endomorphin-1-ol and endomorphin-2-ol both in guinea-pig ileum and in mouse vas deferens (Table 1). ?L-Phe^4-OH?endomorphin-2 was found to be 272 and 433 times less active than its parent compound in mouse vas deferens and ginea-pig ileum respectively. To a lesser extent, similar pattern was observed between the ?L-Pro^4-OH?-morphiceptin and the parent (amidated) morphiceptin, which was approximately 12 times potent than it carboxyl derivative in both organs (Table 1). The dramaticaly loss in potency was observed with ?L-Leu^4-OH?-endomorphin-2 in mouse vas deferens and in the ginea-pig ileum where the IC_{50} values were over the millimolar and micromolar range respectively (Table 1). However, both endomorphin-1-ol and endomorphin-2-ol had shown an equipotent agonist activity in both isolated organs.

TABLE 1. The opioid characteristics of peptides with Tyr-Pro sequence in their N-terminal in longitudinal muscle strip of guinea-pig ileum (GPI) and mouse vas deferens (MVD)

Peptide	GPI		MVD	
	IC_{50} (nM)[a]	Ntx K_e (nM)[b]	IC_{50} (nM)[a]	Ntx K_e (nM)[b]
H-Tyr-Pro-Phe-Pro-NH_2 (Morphiceptin)	**1033.5** (673.1-1586.9) n=6	**0.50** (0.46-0.55) n=6	**858.4** (584.6-1260.4) n=6	**0.14** (0.11-0.19) n=6
H-Tyr-Pro-Phe-Pro-OH (?L-Pro[4]-OH?-Mor)	**13,115** (11,258-15,278) n=4	**1.12** (0.86-1.45) n=4	**10,497** (7,935-13,885) n=6	**0.26** (0.21-0.33) n=6
H-Tyr-Pro-Phe-Phe-NH_2 (EM-2)	**11.0** (7.3-16.7) n=6	**0.41** (0.27-0.61) n=4	**21.36** (14.79-30.85) n=6	**0.27** (0.24-0.29) n=4
H-Tyr-Pro-Phe-Phe-ol (EM-2-ol)	**48.2** (25.8-90.1) n=8	**0.40** (0.27-0.60) n=7	**36.13** (22.16-58.9) n=12	**0.29** (0.20-0.42) n=6
H-Tyr-Pro-Phe-Phe-OH (?L-Phe[4]-OH?-EM-2)	**4,760** (4,180-5,430) n=4	**0.31** (0.24-0.41) n=4	**5,807** (4,444-7,587) n=8	**5.38** (3.06-9.47) n=4
H-Tyr-Pro-Phe-Leu-OH (?L-Leu[4]-OH?-EM-2)	**44,654** (23,654-85,182) n=6	**1.174** (0.743-1.856) n=6	**?1,000,000 (?10^{-3}M)**	
H-Tyr-Pro-Trp-Phe-NH_2 (EM-1)	**14.7** (9.4-23.2) n=9	**0.20** (0.17-0.23) n=4	**31.9** (19.4-52.3) n=14	**0.21** (0.19-0.24) n=4
H-Tyr-Pro-Trp-Phe-ol (EM-1-ol)	**61.2** (45.1-83.0) n=8	**0.31** (0.25-0.37) n=8	**80.6** (57.1-113.6) n=16	**0.42** (0.33-0.53) n=6

Footnotes:

[a] 50% inhibitory concentration; geometric means and 95% confidence intervals are listed

[b] Equilibrium dissociation constant; geometric means and 95% confidence intervals

Table 2 illustrates the effects of endomorphin-related peptides with modifications in positions 1, 2 and 4 tested in mouse vas deferens. ?Dopa[1]?-endomorphin-2 had shown an agonist potency of 20 times less than of the parent compound in the mouse vas deferens (Table 2). In addition, in the same organ, ?? MeDopa[1]?-endomorphin-2 produced an agonist activity of more than 10 ?M (Table 2).

On the other hand, Dmt^{1}-endomorphins had shown an agonist activity approximately 15-25 times higher than of parent peptides either in mouse vas deferens or in ginea-pig ileum (Tables 1, 2, 3). In addition, Dmt^{1}-endomorphins were equipotent in both isolated organs (Table. 3). It should be kept in mind that even natural endomorphins rank among the most potent ? receptor agonists; a further increase in potency provided powerful agonists with IC_{50} values falling into the subnanomolar range. The agonist effect of Dmt^{1}-endomorphins was not only strong but also durable; in contrast to the average washout-recovery cycle characteristic of the majority of opioid agonist peptides in isolated organs, this cycle was as long as 30-50 minutes.

?D-Ser^{2}?-endomorphin-2 was found to display an agonist potency which is equipotent with that of the parent compound in mouse vas deferens (t-test, P=0.690) (Table 2). On the other hand, in the ginea-pig ileum the agonist activity of this peptide was approximately 6 times less than of the parent peptide (Table1, 3) (t-test, p=?0.001). In contrast to ?D-Ser^{2}?-endomorphin-2, ?D-Met^{2}?-endomorphin-2 was less potent than the parent compound either in the mouse vas deferens (t-test, p=0.002) or in the ginea-pig ileum (t-test, p=0.010). In addition this peptide produced a similar agonist activity in both isolated organs (Table1 and 3). ?cycloSer^{2}?-endomorphin-2 was three times less active than the parent peptide wherase ?L-Ser^{2}?-endomorphin-2 was apparently weak agonist in the mouse vas deferens with IC_{50} of two order less than of the parent peptide (Table 2 and 1).

?D-Ser^{2}, D-Ser^{4}?-endomorphin-2 and ?L-Ser^{2}, L-Ser^{4}?-endomorphin-2 displayed an agonist activity of 66 to 187-times less potent than the parent peptide (Table 1 and 2). Moreover, ?Hyp^{2}?–endomorphin-2 had shown an agonist activity of 133 times less than of the parent compound (Table 1 and 2).

TABLE 2. The opioid characteristics of endomorphin-2 and -1 derivative in mouse vas deferens (MVD)

Peptide	IC_{50} (nM)[a]	Ntx K_e (nM)[b]
(2',6' Me)Tyr-Pro-Phe-Phe-NH_2 **Dmt[1]-EM-2**	**0.89** (0.60-1.31, n=5)	**0.52** (0.36-0.76, n=5)
H-Dopa-Pro-Phe-Phe-NH_2 **?3'OH L-tyr[1]?-EM-2**	**440.8** (387.0-502.1, n=4)	**0.37** (0.34-0.40, n=4)
H-? MeDopa-Pro-Phe-Phe-NH_2 **?? Me, 3'OH L-tyr[1]?-EM-2**	**?? 10.000, n= 4**	
H-Tyr-D-Ser-Phe-Phe-NH_2 **?D-Ser[2]?-EM-2**	**32.69** (14.88-71.78, n=8)	**0.23** (0.20-0.26, n=4)
H-Tyr-D-Ser-Phe-D-Ser-NH_2 **?D-Ser[2], D-Ser[4]?-EM-2**	**1,395** (1,211-1,607, n=4)	**0.39** (0.32-0.47, n=3)
H-Tyr-Ser-Phe-Phe-NH_2 **?L-Ser[2]?-EM-2**	**2,336** (1,719-3,176, n=4)	**0.28** (0.24-0.32, n=4)
H-Tyr-Ser-Phe-Ser-NH_2 **?L-Ser[2], L-Ser[4]?-EM-2**	**3,938** (3,593-4,316, n=4)	**0.26** (0.22-0.30, n=4)
H-Tyr-cycloSer-Phe-Phe-NH_2 **?cycloSer[2]?-EM-2**	**67.2** (58.8-77.1, n=4)	**0.35** (0.27-0.44, n=4)
H-Tyr-HyPro-Phe-Phe-NH_2 **?Hyp[2]?-EM-2**	**2,840** (2,251-3,582, n=4)	**0.25** (0.18-0.34, n=4)
H-Tyr-D-Met-Phe-Phe-NH_2 **?D-Met[2]?-EM-2**	**81.35** (38.66-171.17, n=7)	**0.38** (0.34-0.42, n=4)
(2',6'Me)Tyr-Pro-Trp-Phe-NH_2 **Dmt[1]-EM-1**	**0.87** (0.49-1.57, n=12)	**0.61** (0.41-0.90, n=5)

Footnotes:

[a] 50% inhibitory concentration; geometric means and 95% confidence intervals are listed

[b] Equilibrium dissociation constant; geometric means and 95% confidence intervals

TABLE 3. The opioid characteristics of prominent endomorphin-2 and -1 derivatives in longitudinal muscle strip of guinea-pig ileum (GPI)

Peptide	IC_{50} (nM)[a]	Ntx K_e (nM)[b]	GPI/ MVD[c] Potency Ratio
Dmt[1]-EM-2	**0.70** (0.48-1.01, n=4)	**0.26** (0,06-1.21, n=3)	1.27
?D-Ser[2]?-EM-2	**71.69** (60.87-84.45, n=5)	**0.63** (0.41-0.95, n=5)	0.46
?D-Met[2]?-EM-2	**81.68** (44.27-150.68, n=5)	**0.57** (0.40-0.80, n=5)	0.99
Dmt[1]-EM-1	**0.81** (0.47-1.42, n=6)	**0.57** (0.30-1.08, n=5)	1.07

Footnotes:

[a] 50% inhibitory concentration; geometric means and 95% confidence intervals are listed

[b] Equilibrium dissociation constant; geometric means and 95% confidence intervals

[c] The IC_{50} in mouse vas deferens divided by the one in guinea-pig ileum

In the series of enkephalin analogs the amidated derivative of the ?-opioid prototype selective agonist peptide (DAMGO), DAMGA was approximately 3-4 times more potent than the parent peptide either in the mouse vas deferens or in the guinea-pig ileum bioassay; the IC_{50}-values are presented in Table 4. However, both DAMGO and DAMGA were found to have an IC_{50} 2 times higher in the mouse vas deferens than in the guinea-pig ileum. In contrast, ?D-Ala[2]?-leu-enkaphalin proved to be approximately 60 times more potent in the mouse vas deferens than in the ginea-pig ileum (Table 4).

TABLE 4. The opioid characteristics of enkephalin derivatives, (D-Ala^2, Leu-enkephalin, DAMGO and its Gly^5-NH_2 congener) in longitudinal muscle strip of guinea-pig ileum (GPI) and mouse vas deferens (MVD)

Peptide	GPI		MVD	
	IC_{50} (nM)[a]	Ntx K_e (nM)[b]	IC_{50} (nM)[a]	Ntx K_e (nM)[b]
?D-Ala^2?Leu-enkephalin	**72.8** (57.6-91.4) n=4	**0.54** (0.35-0.83) n=4	**1.18** (0.97-1.43) n=6	**6.20** (4.96-7.74) n=6
DAMGO	**29.2** (17.5-48.6) n=12	**0.30** (0.27-0.33) n=5	**63.96** (46.45-88.07) n=9	**0.33** (0.29-0.37) n=6
DAMGA	**7.99** (4.95-12.9) n=6	**0.31** (0.25-0.39) n=4	**18.32** (14.11-23.79) n=13	**0.40** (0.26-0.59) n=6

Footnotes:

[a] 50% inhibitory concentration; geometric means and 95% confidence intervals are listed

[b] Equilibrium dissociation constant; geometric means and 95% confidence intervals

B.) Determination of receptor constants for ?-opioid receptor agonists in mouse vas deferens.

From the pilot experiments, the chosen concentration of ?-funaltrexamine was $5x10^{-7}$ M. It caused 34.1 ? 1.4 (n=56, arithmetic mean ? S.E.M.) inhibition, with a moderate tendency of recovery throughout the 30 min exposure at the endpoint. After 40-60 minutes and 8-12 washes there was a recovery to 94.9 ? 1.9% of control; no exclusion was necessary since recovery was higher than 80% in each experiment. Figure 4 illustrates the dose response curves before and after exposure to $5x10^{-7}$ M ?-FNA for endomorphin-1 and endomorphin-1-ol (panel A), DAMGA and DAMGO (panel B) in MVD. Concentration-effect curves for certain compounds such as endomorphin-2 and endomorphin-2-ol, morphine and normorphine obtained in the same preparation and under the same conditions are presented in fig. 5, 6 respectively.

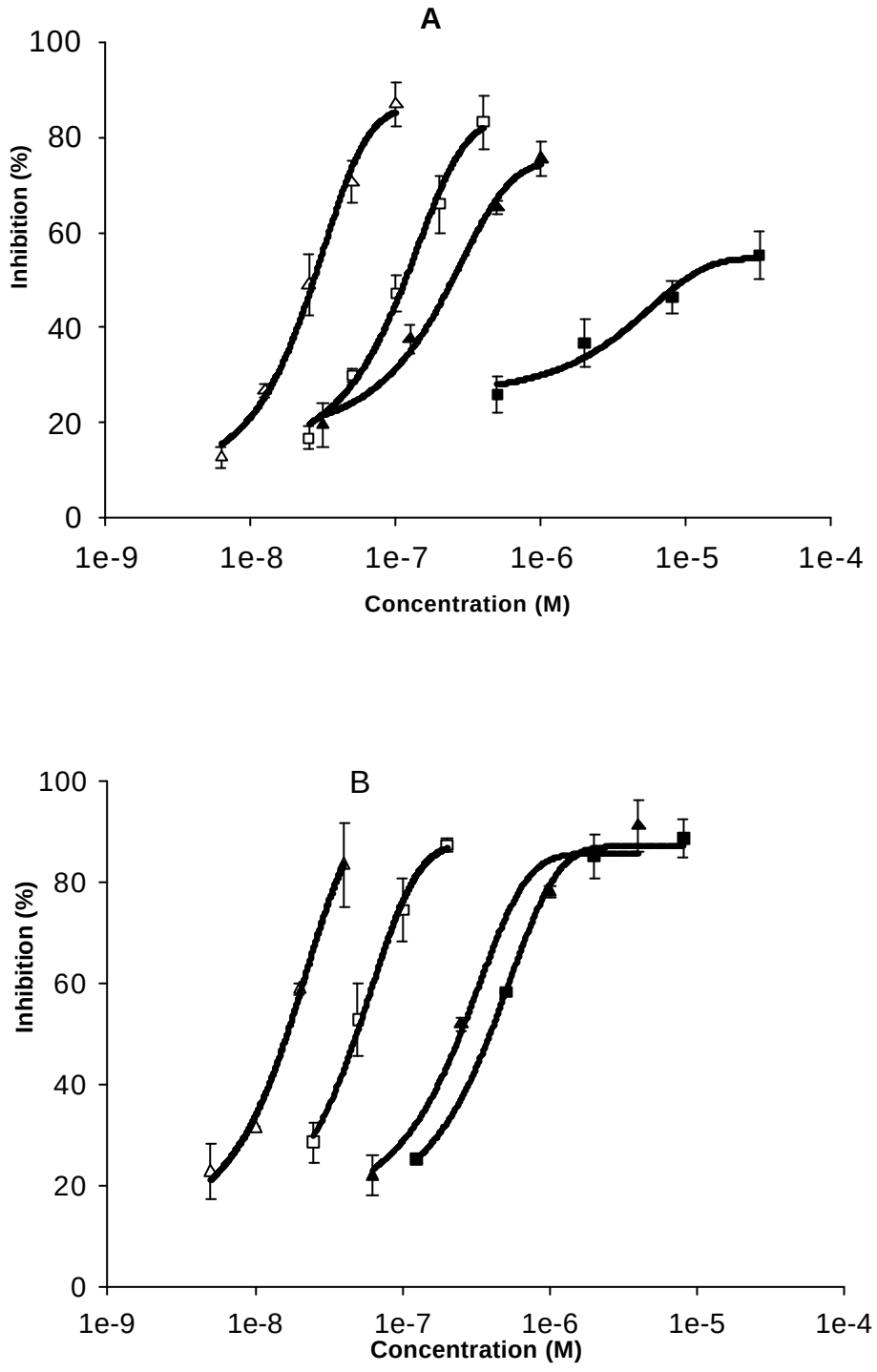


Fig. 4. The inhibitory dose-response curves of endomorphin-1 (? , ?) and endomorphin-1-ol (?, ¦) (panel A, n=6); DAMGA (? , ?) and DAMGO (?, ¦) (panel B, n=4 and 5 respectively) in the mouse vas deferens. Points represent the arithmetic mean, vertical lines the S.E. Symbols: open: values obtained before ?-FNA (control); dark: values obtained after 30 min exposure to $5x10^{-7}$ M ?-FNA.

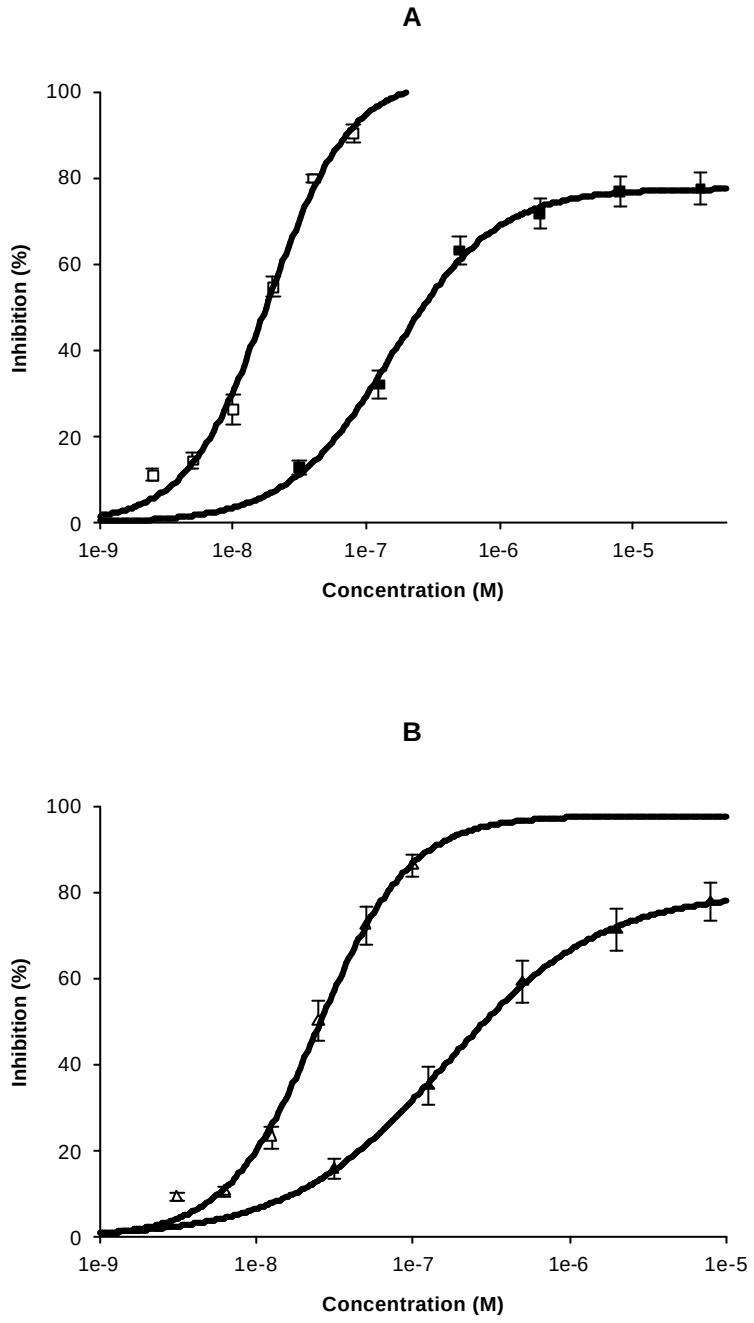


Fig. 5. The inhibitory dose-response curves of ?- opioid receptor agonists in mouse vas deferens before and after ?-funaltrexamine treatment.

Panels: A: endomorphin-2 (n=5); B: endomorphin-2-ol (n=6). Points represent the arithmetic mean, vertical lines the S.E. Symbols: open: values obtained before ?-FNA (control); dark: values obtained after 30 min exposure to $5x10^{-7}$ M ?-FNA.

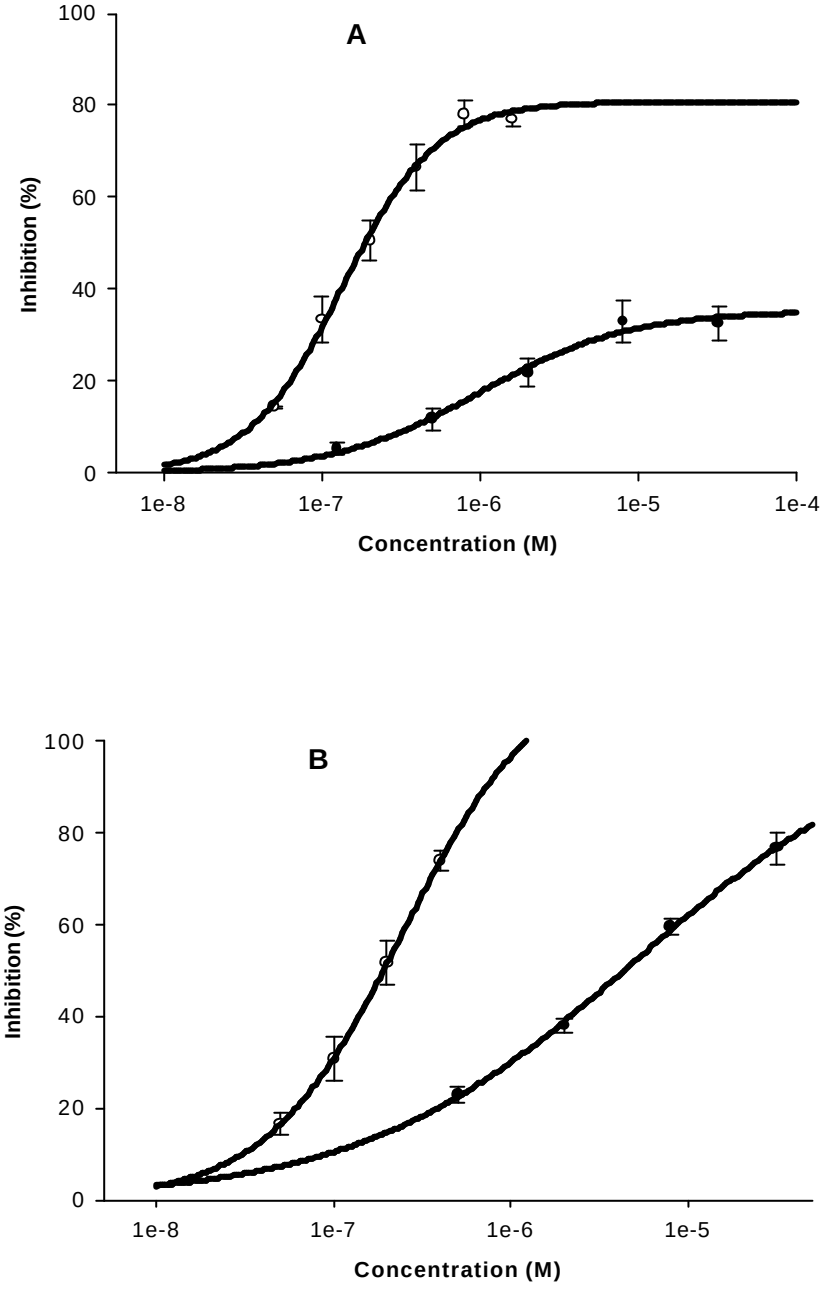
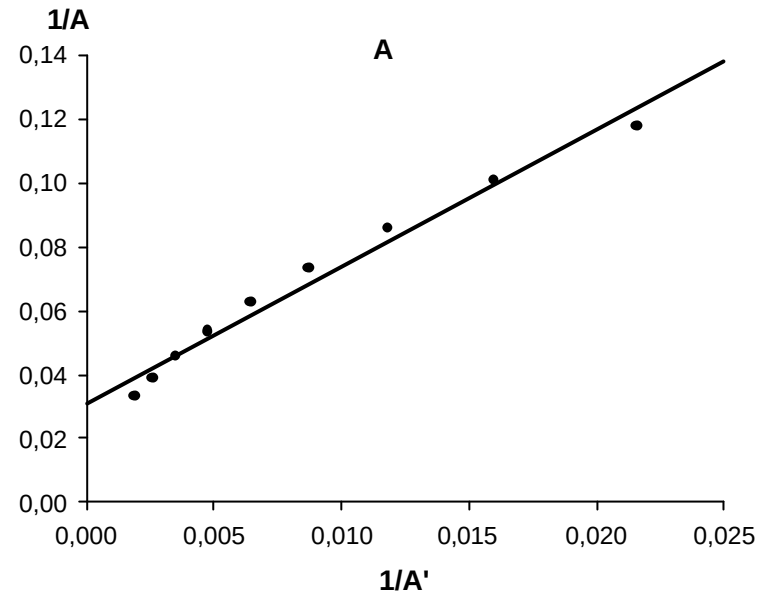


Fig. 6. The inhibitory dose-response curves of ?- opioid receptor agonists in mouse vas deferens before and after ?-funaltrexamine treatment. Panels: A: Morphine (n=4); B: Normorphine (n=4). Points represent the arithmetic mean, vertical lines the S.E. Symbols: empty before (control) and full after $5x10^{-7}$ M ?-FNA treatment.

Pretreatment of the vasa with $5x10^{-7}$ M ?-FNA caused a rightward shift, slope reduction and in some cases a considerable E_{max} reduction of agonist dose response curves. Nevertheless, the scope of these changes was different for the various subsets of agonists.

To obtain receptor constants for the agonists according to the method described by Furchgott and Bursztyn ?42?, first equieffective concentrations were chosen and plotted as reciprocals for individual, paired experiments (for details see Methods); such plots are shown in figure 7 for endomorphin-1 (panel A), endomorphin-1-ol (panel B) and in figure 8 for DAMGO (panel A) and DAMGA (panel B).

From the double reciprocal plots and their dose response curves the control IC_{50} values, K_A, q and K_A/ IC_{50} ratio were obtained (Table 5).



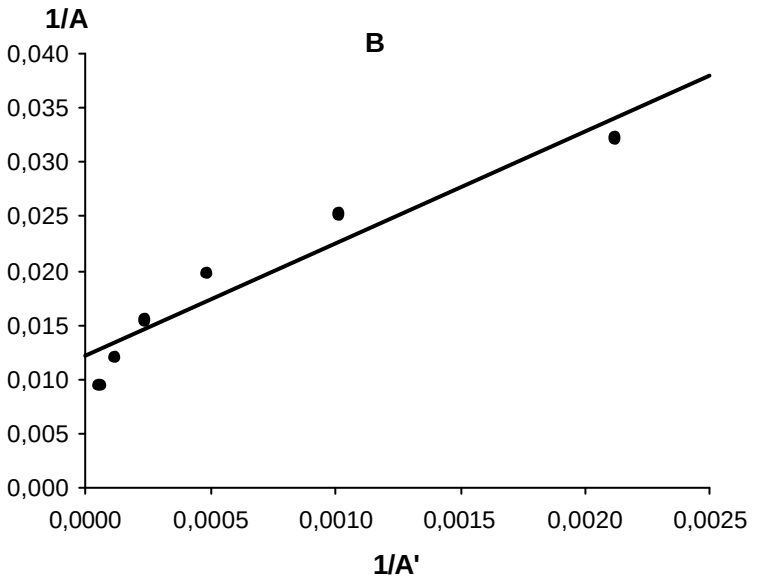
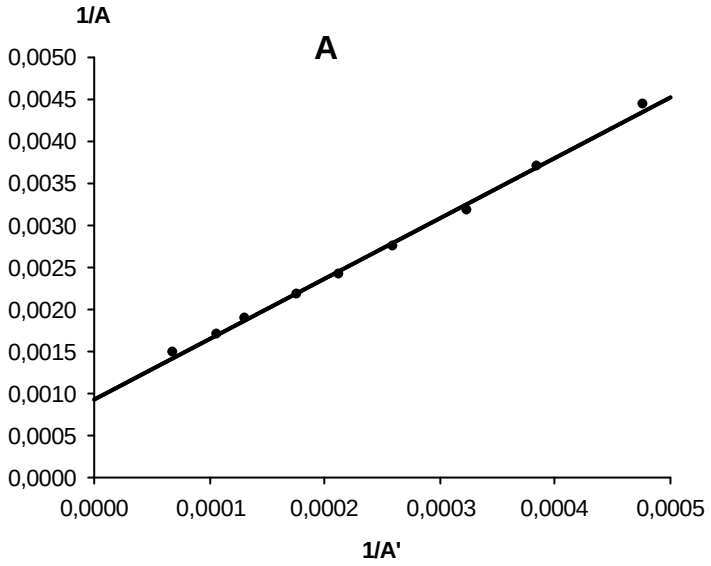


Fig. 7. Double reciprocal plots of equieffective concentrations before and after treatment the tissue with ?-FNA for endomorphin-1 (panel A), endomorphin-1-ol (panel B) in MVD (single experiment). 1/A and 1/A' are the reciprocal of equieffective agonist concentrations before and after ?-FNA treatment respectively.



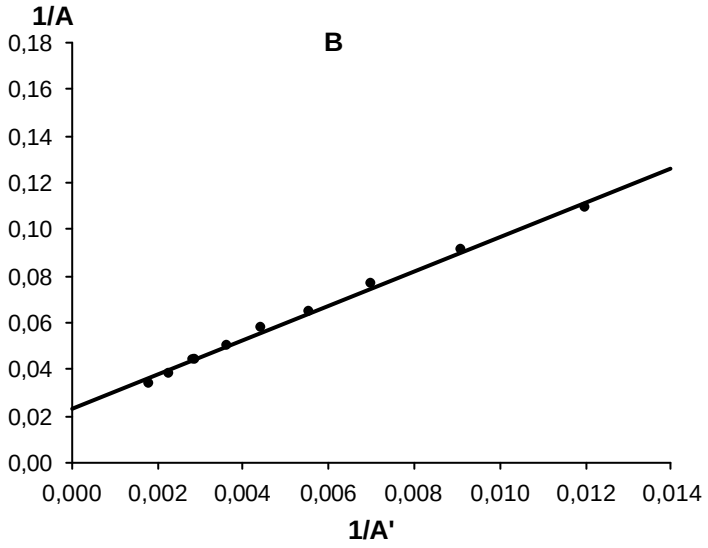


Fig. 8. Double reciprocal plots of equieffective concentrations before and after treatment the tissue with ?-FNA for DAMGO (panel A) and DAMGA (panel B) in the MVD (single experiment). 1/A and 1/A' are the reciprocal of equieffective agonist concentrations before and after ?-FNA treatment respectively.

TABLE 5. The receptor constants of some ? -receptor agonists in isolated MVD

No	Compound	(n)[a]	IC_{50}(nM)[b]	K_A (nM)[c]	q (%)[d]	K_A/IC_{50}
1	Normorphine	4	**178.3** (139.6-227.8)	**1,748** (1,542-1,981)	**14.4** (11.8-18.9)	**9.80** (8.11-11.8)
2	Morphine	4	**219.3** (158.4-280.9)	**685.4** (323.6-1,452)	**12.7** (7.94-20.3)	**3.25** (1.93-5.46)
3	DAMGO	5	**50.1** (41.5-60.6)	**728** (489-1,220)	**16.9** (12.5-22.8)	**15.4** (10.3-23.2)
4	DAMGA	4	**18.50** (16.66-20.54)	**355** (274-461)	**14.1** (11.0, 13.5)	**19.2** (14.6-25.2)
5	Morphiceptin	4	**2,636** (2,330-2,983)	**36,245** (28,967-45,350)	**12.5**[e] (11.6-13.5)	**13.8** (9.82-19.3)
6	EM-2	5	**17.5** (15.3-20.0)	**117.2** (74.8-183.5)	**17.5** (15.3-20)	**6.69** (4.21-10.6)
7	EM-2-ol	6	**26.3** (20.4-34.0)	**92.6** (68.2-125.8)	**27.9** (22.2-35.2)	**3.51** (2.53-4.86)
8	?D-Ser[2]?-EM-2	4	**71.1** (60.2-83.9)	**2,220** (1,462-3,370)	**3.6** (2.6-4.9)	**29.7** (22.3-39.6)
9	?D-Met[2]?-EM-2	4	**40.7** (21.8-76.0)	**465.3** (319.5-677.5)	**11.2** (7.68-16.3)	**11.2** (9.07-13.8)
10	EM-1	6	**23.7** (19.0-29.6)	**38.4** (19.2-77.0)	**45.1** (33.2-61.1)	**1.62** (0.94-2.81)
11	EM-1-ol	6	**105.31** (80.52-137.74)	**632** (465-859)	**14.0** (10.3-18.9)	**5.58** (4.66-6.68)
12	Dmt[1]-EM1-1	6	**1.41** (1.08-1.84)	**4.83** (2.66-7.09)	**30.0**[f] (19.9-45.5)	**3.01**[g] (1.57-6.08)

Footnotes

[a]Number of experiments

[b]50% inhibitory concentrations; geometric means and 95% confidence intervals

[c]Apparent dissociation constant of agonist; geometric means and 95% confidence intervals

[d]Active residual receptor fraction; geometric means and 95% confidence intervals

STATISTICS

q: for compound "10" q is different at p<0.01 from all the others
for compound "12" q is different from 4,8,9,11 (p<0.01) and 1,2,3,5 (p<0.05)
for compound "7" q is different from 4,9,11 (p<0.05) and 8 (p<0.01)
for compound "8" q is different from 7,10,12 (p<0.01)

K_A/IC_{50}: for compound "8" is different at p<0.01 from all but "4"
for compound "3" is different from 2,7,8,10,11,12 (p<0.01) and 6 (p<0.05)
for compound "4" is different from 2,7,10,11,12 (p<0.01) and 6 (p<0.05)
for compound "5" is different from 8,10,12 (p<0.01) and 2,7 (p<0.05)

Likewise, we determined the receptor constants for the rest of agonists in Table 5. The efficacies of drugs were calculated as the ratio of K_A over IC_{50}. The IC_{50} were extended between 1.41 and 2,632 nM whereas the K_A values between 4.83 and 36,245 nM (Table 5); these sets of data were not analyzed statistically. The efficacy values were ranged between 1.62 and 29.7 (Table 5). For most of agonists, the fractions of viable receptors left in the tissues after ?-FNA treatment (q) fall into the 11.2-17.5% range whereas significantly higher values for three agonists and very low value for one (Table 5).

?D-Ser^2?-endomorphin-2 had apparently a prominent high efficacy value among the other agonists. This tendency prompted us to check its validity in the rat vas deferens, which contains a ?-opioid receptor-like receptor supply with very low receptor reserve ?155?. Therefore, only high-efficacy ?-opioid receptor agonists can be expected to be effective in this preparation. DAMGO was found to be highly effective agonist whereas ?D-Ser^2?-endomorphin-2 was a weak agonist in isolated rat vas deferens (Al-Khrasani and Rónai, unpublished). The possible reasons for this difference will be treated in detail in the discussion part. Thus, when we assessed the rank order of agonist efficacies, ?D-Ser^2?-endomorphin-2 was excluded from the evaluation. Taking the efficacy of DAMGO as 100, the rank order of the relative efficacies of the agonists were DAMGA? DAMGO ? morphiceptin? ?D-Met^2?-EM-2 ? normorphine ? EM-2 ? EM-1-ol ? Dmt^1-EM-1 ? morphine ? EM-2-ol ? EM-1 (Table 6). Statistically, the results of this analysis suggest that DAMGO, DAMGA, morphiceptin behave as the full agonists, ?D-Met^2?-EM-2 and normorphine are possibly full agonists whereas EM-2, EM-1-ol, Dmt^1-EM-1, morphine, EM-2-ol and EM-1 as the partial agonists.

TABLE 6. The relative efficacies of some ?-receptor agonists in the mouse vas deferense, based on the K_A/IC_{50} ratios

Agonist	Relative efficacy[a]	(n[b])
DAMGO	100[c]	
DAMGA	118.6 ? 15.9	4
Morphiceptin	86.9? 15.3	4
?D-Met?[2]-EM-2	68.1? 7.7	4
Normorphine	59.5? 5.8	4
EM-2	44.1? 8.7?	5
EM-1-ol	36.8? 3.1??	4
?Dmt[1]?-EM-1	23.1? 6.3??	6
Morphine	22.1? 6.1??	4
EM-2-ol	22.1? 3.2??	6
EM-1	11.1? 2.1??	6

Footnotes

[a]The efficacy of DAMGO was taken as 100. Arithmetic mean ? S.E.M

[b]Number of experiments

[c]The K_A/IC_{50} value for DAMGO was 16.8 ? 2.8 and n=4

? p?0.05 vs DAMGA; ?? p?0.05 vs. both DAMGO and DAMGA by ANOVA followed by LSD test.

The reduction in the effectiveness of ?-opioid receptor agonist deltorphin-II was moderate though statistically significant (88.3 ? 1.5 % inhibition before vs 67.7 ? 2.4 % after ?-funaltrexamine exposure at 10^{-9} M concentration of agonist; p?0.05, n=16, paired "t" test).

THE MODULATORY EFFECTS OF ENDOMORPHINS AND DAMGO ON THE FIELD STIMULATION-INDUCED ^{3}H-NOREPINEPHRINE RELEASE FROM ADULT RAT NUCLEUS TRACTUS SOLITARII-DORSAL MOTOR VAGAL NUCLEUS SLICES

Applying two electrical field stimulations 30 min apart (S1 and S2) to induce ^{3}H-NE release from adult rat nucleus tractus solitarii-dorsal motor vagal nucleus (NTS-DVN) slices results in S_2 over S_1 ratio of 1.05 value for control (Table.7 and fig 9, 10).

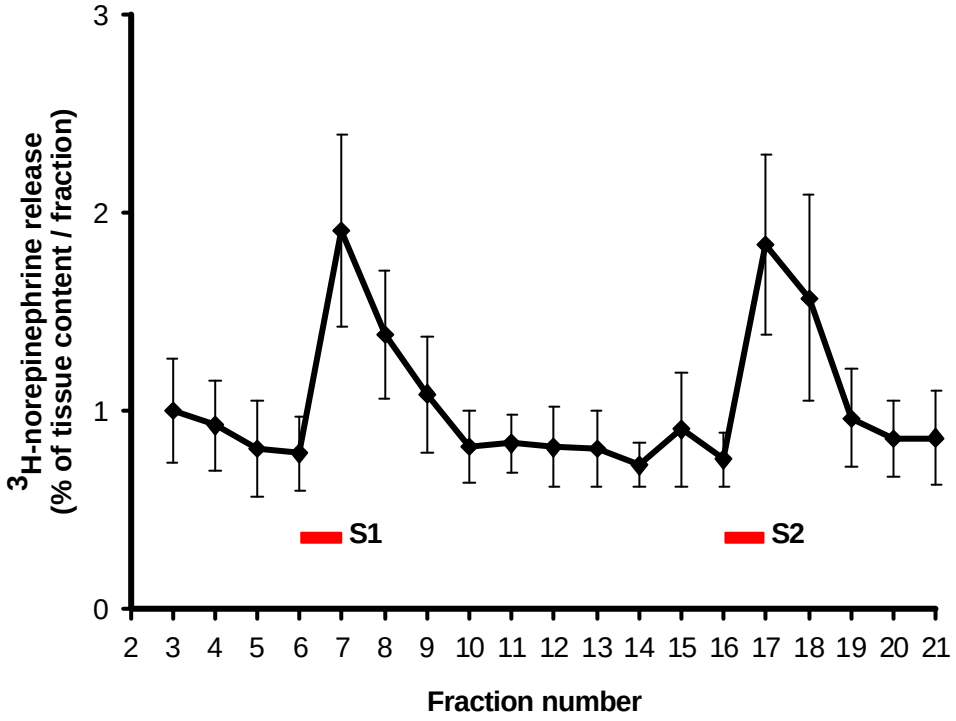


Fig. 9. The effects of field electrical stimulation on ^{3}H-NE release from rat NTS-DVN slices (control, n=4). Stimulations indicated by S1 and S1 (red bars). Points represent the mean ? S.E.M.

The ?$_2$-adrenoceptor receptor agonist clonidine (10^{-6}M) reduced this ratio to 0.34 % (Table 7 and fig 10).

DAMGO caused a dose dependent and naloxone-reversible inhibitory effect on electrical stimulus induced release of the ^{3}H-NE from (NTS-DVN) slices (fig.10 and 11, Table 7).

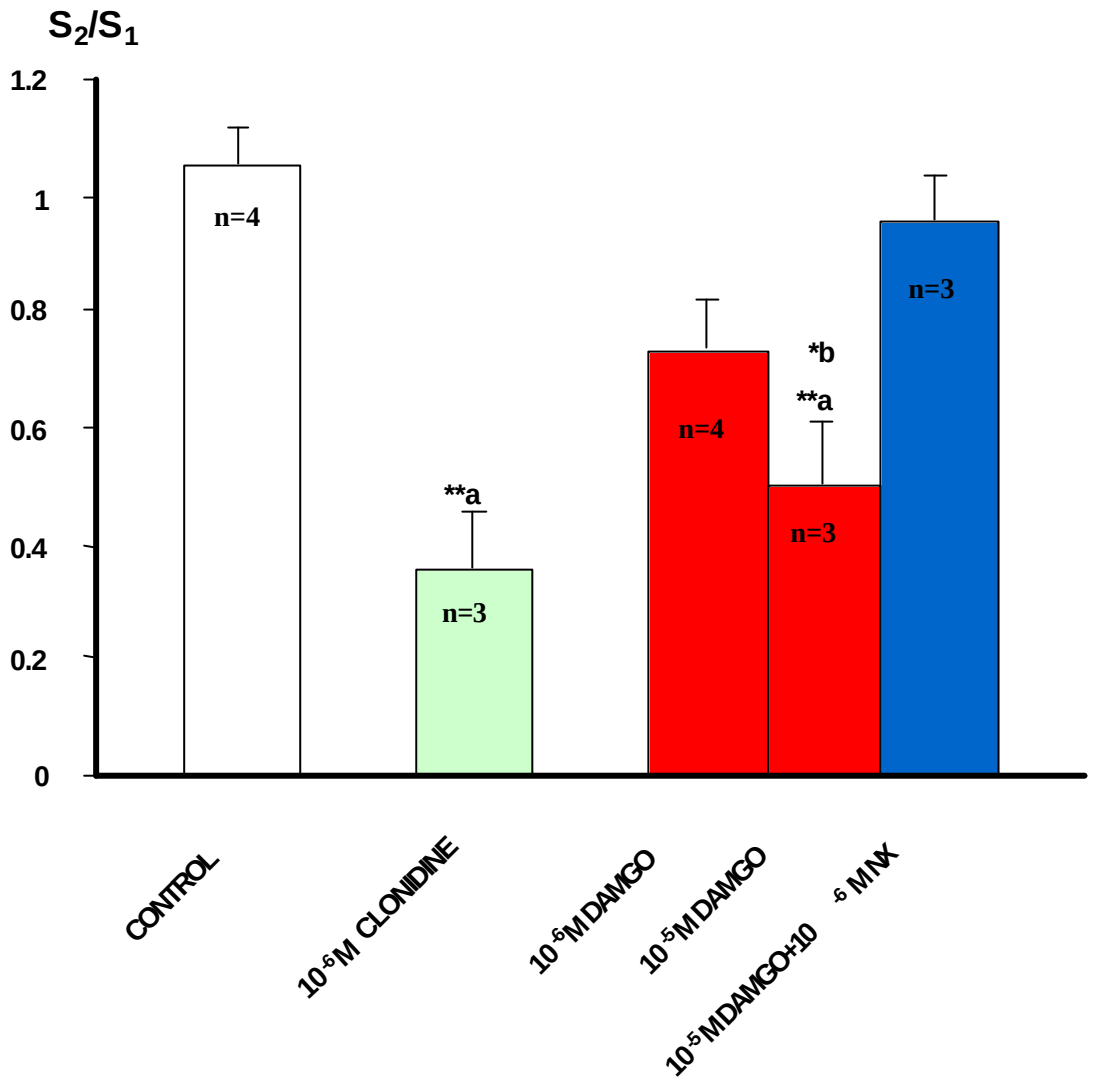


Fig. 10. The effects of clonidine and DAMGO on the stimulation-induced ^{3}H-NE release from adult rat NTS-DVN. Data are expressed as mean? S.E.M. * p?0.05; ** p?0.01.

S_2/S_1: the ratio of area under the curve after stimulation S2 and S1, respectively.

a- compared to the control (white column)

b- compared to 10^{-6} DAMGO (3^{d}, red column)

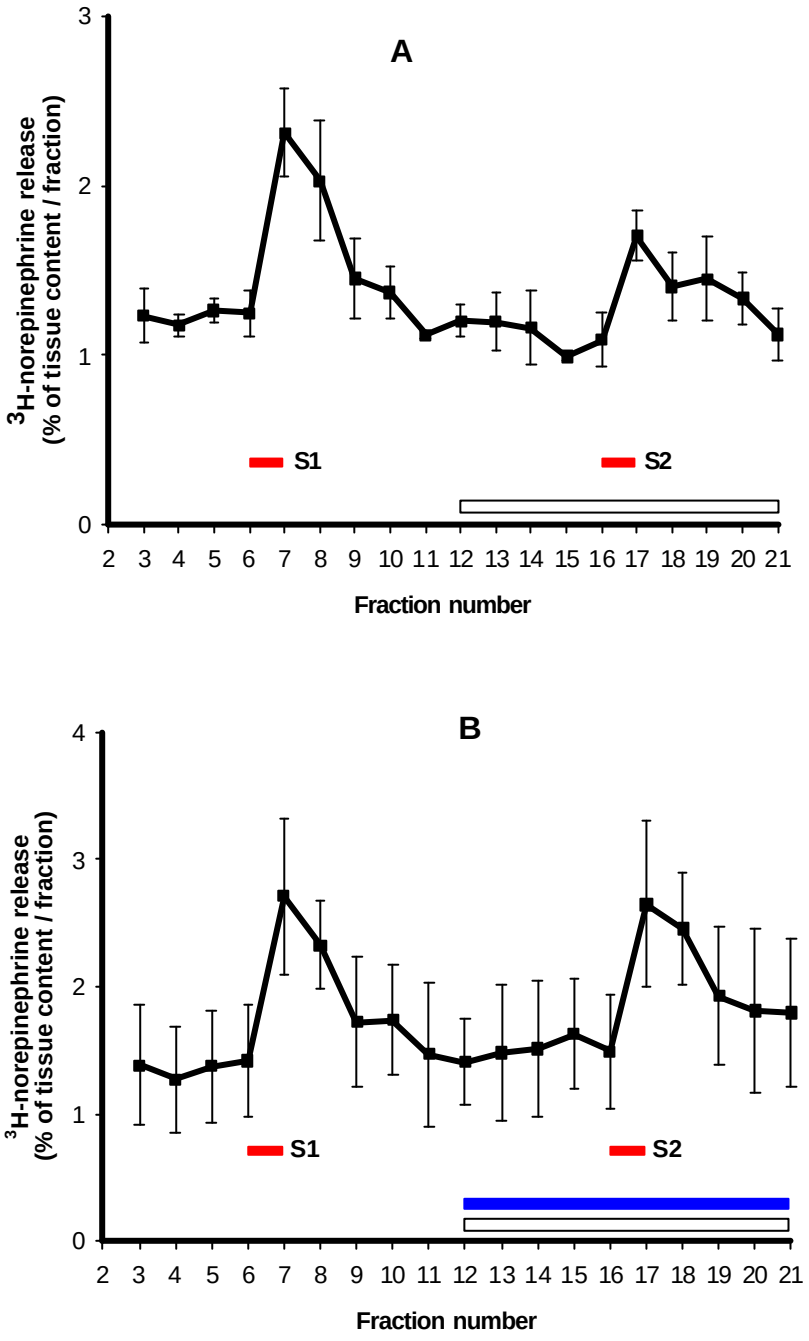


Fig. 11. The naloxone-reversible inhibitory effect of DAMGO on the stimulation-induced ^{3}H-norepinephrine release from rat nucleus tractus solitarii-dorsal motor vagal nucleus slices. Panel A: 10^{-5} M DAMGO (open bar), panel B: 10^{-5} M DAMGO+ 10^{-6} M NX (open bar + dark blue bar). Points represent the arithmetic mean, vertical lines, the S.E.values obtained in three independent experiments. Stimulation cycles indicated by S1 and S2 (red bars).

In the absence of dipeptidyl aminopeptidase (DAP-IV) inhibitor (Diprotin A) endomorphin-1 at the concentrations of 10^{-6} M and 10^{-5} M caused S_2/S_1 ratios of 0.81 and 0.64 respectively whereas these figures for endomorphin-2 0.71 and 0.59. The inhibitory effect of both peptides at the concentration of 10^{-6}M was enhanced in the presence of diprotin A (fig.12 and Table 7). However the presence of diprotin A did not influence the inhibitory effect of endomorphins at the concentration of 10^{-5}M. This effect appeared to have a ceiling at an S_2/S_1 of 0.60 (Table 7).

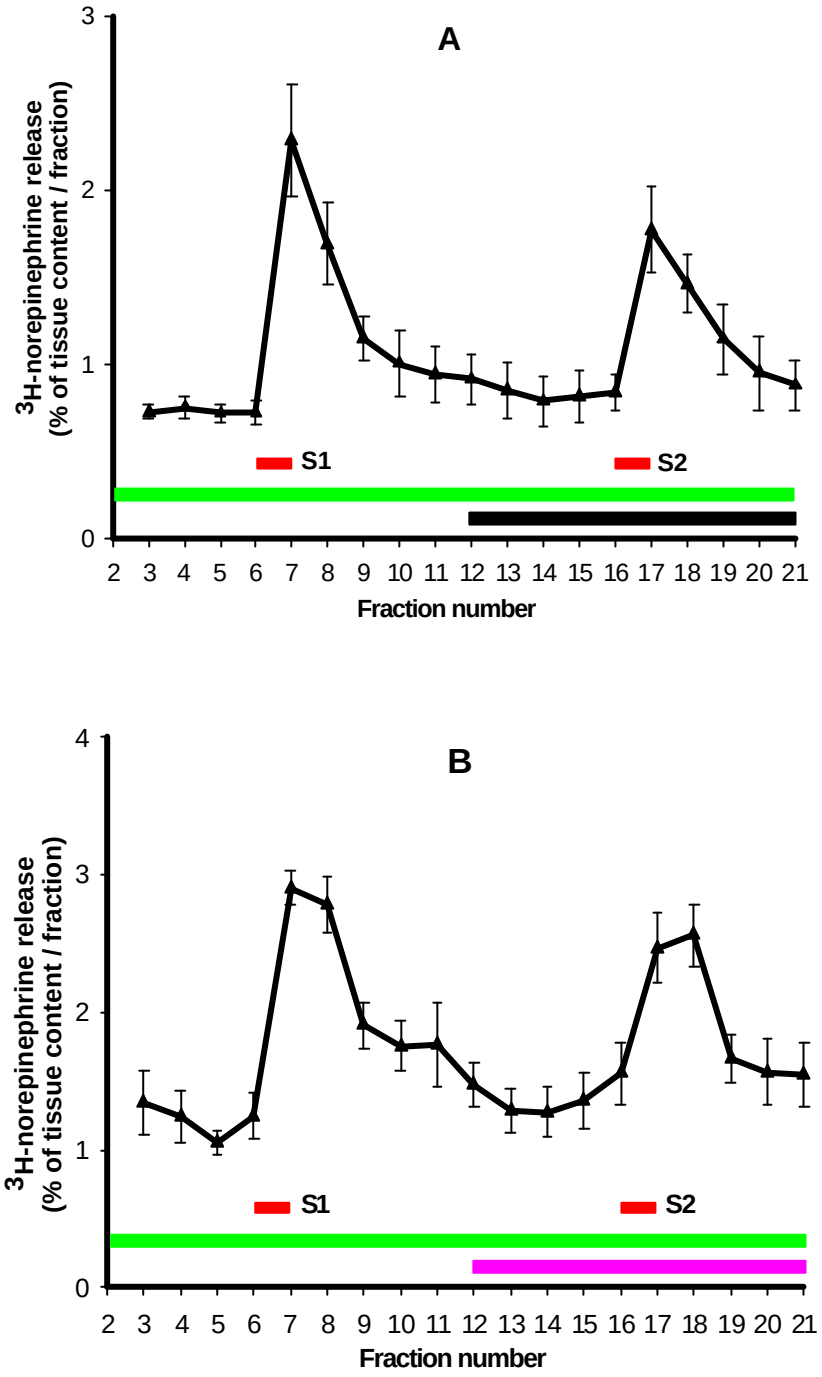


Fig.12 The inhibitory effect of endomorphins on the stimulation-induced ^{3}H-norepinephrine release from adult rat nucleus tractus solitarii-dorsal motor vagal nucleus slices in the presence of DAP-IV enzyme inhibitor diprotin A (green bar). Panel A: 10^{-5}M endomorphin-1 (black bar); panel B: 10^{-5}M endomorphin-2 (pink bar). Slices were stimulated at S1 and S2 (red bars). Points represent the arithmetic mean, vertical lines, the S.E. Values obtained in four independent experiments.

Diprotin A slightly but significantly enhanced the stimulation-induced ^{3}H-NE release, the AUC (S_1) values being 2.26? 0.18 (n=32) in the absence and 3.42?0.41(n=16) in the presence of enzyme inhibitor ($p<0.01$). The presence of diprotin A added 12 min before the second stimulation did not effect the S_2/S_1 ratio.

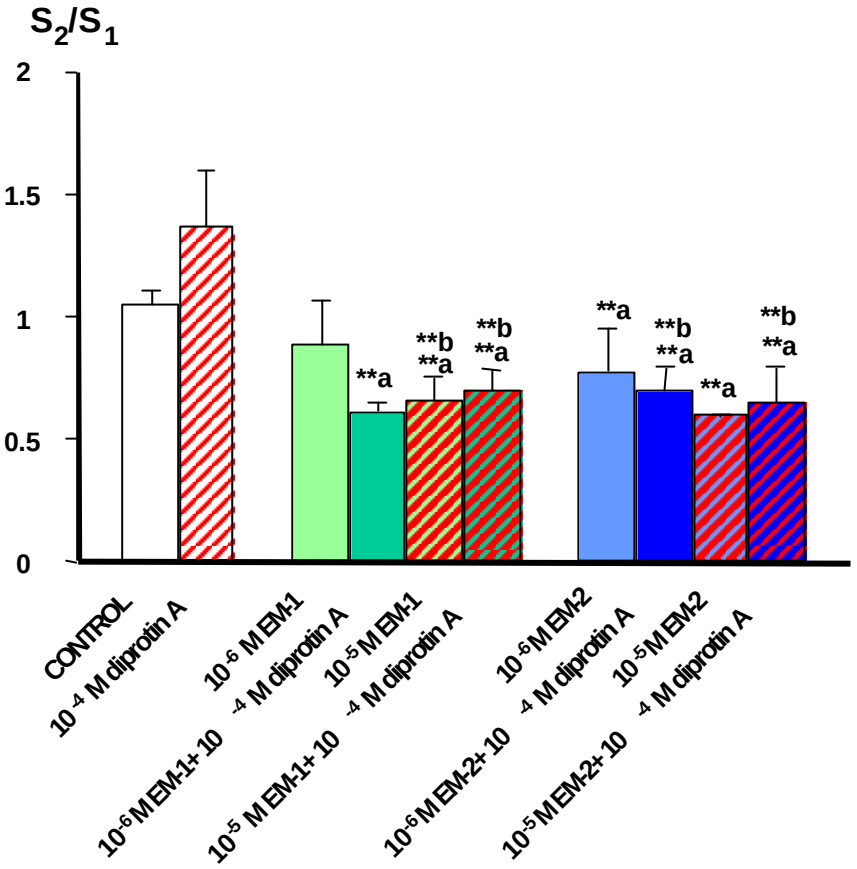


Fig. 13. Summary of the S_2/S_1 ratios in the presence and absence of Diprotin A in adult rat NTS-DVN slices.

Data are presented as mean ?S.E.M obtained from 4 animals with exception 10^{-6}M EM-1 (n=6). ** p?0.01;

S_2/S_1: the ratio of area under the curve after stimulation S2 and S1, respectively.

a- compared to the control (white column)

b- compared to diprotin A (2nd, hatched column)

TABLE 7.The effect of ?-opioid receptor agonists and clonidine on the stimulation-induced release of ^{3}H-norepinephrine from adult rat nucleus tractus solitarii-dorsal vagal nucleus slices

No	Drug[a]	Conc. (M)	N	S_2/S_1[b]	Statistics LSD[c]-test
1	None	----	4	**1.047** (0.946-1.159)	-------
2	Clonidine	10^{-6}	3	**0.336** (0.230-0.490)	p?0.01 vs. 1
3	DAMGO	10^{-6}	4	**0.726** (0.590-0.892)	p?0.01 vs. 2
4	DAMGO	10^{-5}	3	**0.483** (0.355-0.657)	P?0.01 vs. 1; p?0.05vs. 3
5	DAMGO +Naloxone	10^{-5} 10^{-6}	3	**0.950** (0.840-1.073)	p?0.01 vs. 4
6	Diprotin A	10^{-4}	4	**1.249** (0.922-1.816)	
7	EM-1	10^{-6}	6	**0.806** (0.576-1.258)	p?0.01 vs. 2
8	EM-1 +Diprotin A	10^{-6} 10^{-4}	4	**0.613** (0.594-0.683)	p?0.01 vs. 1; p?0.01 vs. 6; p?0.01 vs. 2
9	EM-1	10^{-5}	4	**0.642** (0.512-805)	p?0.01 vs. 1; p?0.01 vs. 2
10	EM1 +Diprotin A	10^{-5} 10^{-4}	4	**0.675** (0.547-0.837)	p?0.01 vs. 1; p?0.01 vs. 6; p?0.01 vs. 2
11	EM-2	10^{-6}	4	**0.710** (0.465-1.085)	p?0.01 vs. 1; p?0.01 vs. 2
12	EM-2 +Diprotin A	10^{-6} 10^{-4}	4	**0.665** (0.500-0.886)	p?0.01 vs. 1; p?0.01 vs. 6; p?0.01 vs. 2
13	EM-2	10^{-5}	4	**0.590** (0.571-0.609)	p?0.01 vs. 1; p?0.05vs. 2
14	EM-2 +Diprotin A	10^{-5} 10^{-4}	4	**0.557** (0.309-1.009)	p?0.01 vs. 1; p?0.01 vs. 6; p?0.05vs. 2

Footnotes

[a]Drugs were added 15 min before S2 with the exception of diprotin A which was added 28 min before S1.

[b]The ratio of area under the curve after stimulation S2 and S1, respectively, geometric means and 95% confidence intervals are listed.

[c]ANOVA followed by Least Significant Differences test.

DISCUSSION

ANALYSIS OF OPIOID PROPERTIES IN ISOLATED ORGANS

A.) General opioid pharmacology of endomorphin-related peptides

Since one of the major aims was to determine the opioid characteristics of novel endomorphin derivatives in isolated organs, the principle of measurement and the "thumb rules" of characterization should be discussed first. The assay systems were neurally (field) stimulated smooth muscle preparations containing presynaptically located inhibitory ?, ? or ? type of opioid receptors. The MVD contains all the three major opioid receptor types whereas GPI contains functional ? and ? types [9; 82; 86; 175]. Agonist potencies can be characterized by the presynaptic inhibitory effects, expressed most conveniently in terms of 50 % inhibitory concentrations (IC_{50}). The receptor types where the agonists exert their actions could be determined by analyzing the interaction with the moderately ? receptor type-preferring competitive opioid antagonist naltrexone. The affinity of naltrexone to the preferred i.e. ? receptor type can be characterized by a K_e falling into the 0.2-0.6 nM range [48; 139]. The affinity of antagonist towards both the ? and ? opioid receptors is 20-30 times lower [48; 136]. Therefore, if an agonist-naltrexone interaction in MVD where all the three opioid receptor types are present yields a naltrexone K_e in the 0.2-0.6 nM range it can be declared that the agonist exerted its action at the ?-opioid receptors in the concentration range used in the interaction analyses. If the antagonist K_e is conspicuously and also statistically different from the indicated range then the agonist is likely to act also at other (i.e. ? or ?) opioid receptor types.

Since the MVD contains a dominant ? receptor pool with a considerable spare fraction whereas GPI lacks functional ? receptors, ?-receptor-preferring agonists tend to be more potent in MVD than in GPI [86; 82]. On the other hand, the spare ? and ? receptor pool is much higher in GPI than in MVD [100]. Consequently, ? receptor-preferring agonists [89] and ? partial agonists are likely to be more potent in GPI than in MVD.

The novel endomorphin derivatives were modified in positions 1, 2 and at the C-terminus. C-terminal free carboxylic function in the endomorphin series, similarly to enkephalin-related peptides [137; 138] and morphiceptin, reduced agonist potency and

also the selectivity at ?-opioid receptors. In the case of EM-2 with free carboxylic terminus, in contrast to the morphiceptin-related analogous pair, there was a clear indication of ? receptor type selection. One should recall that Tyr-Tic-Phe-Phe-OH (TIPP, ?114; 146?) has been reported to possess selective ?-opioid receptor antagonist properties. Aromatic moiety in position 4 is more advantageous for opioid activity than an aliphatic one (Leu, Ser, D-Ser) or proline. Alcoholic C-terminal function, similarly to the previous experience acquired in the enkephalin-analogue series, provides almost identical advantages in potency and selectivity at ?-opioid receptors as compared to C-terminal amidation.

Introduction of a hydroxyl into position 3' of tyrosine[1] resulted in moderate loss in potency. If, however, ?-methylation was accompanied by the introduction of a hydroxyl into position 3' of aromatic ring, the reduction in agonist potency was approximately of 500-times. One may surmise that ?-methylation may cause an unfavorable change in the steric position of one of phenolic hydroxyl in the aromatic ring.

2', 6'-dimethylation on Tyr[1], presumably partly due to an increased hydrophobicity, partly due to an induced conformational constraint on the side chain is known to enhance the affinity of resulting structures to more than one type of opioid receptors ?5; 14; 21; 47; 113; 126; 141; 142?. In the endomorphin series, it resulted in analogues with subnanomolar IC_{50} values and, as judged from the slow washout pattern, in slow dissociation kinetics.

Substitution of Pro[2] by Hyp or Ser cause a 100-fold loss of potency. On the other hand, cycloserine, D-Ser or D-Met substitution causes no or only a minor loss in potency. One should realize that the tetrapeptides containing an aliphatic D-amino acid in position 2 cannot be designated as endomorphin analogues in the strictest sense; the opioid structural motif bears a much closer resemblance to amphibian skin opioid peptides.

In general and for the purposes of next discussion section in particular, it should be pointed out that, as judged from their interaction with naltrexone, all endomorphin analogues containing C-terminal amide or alcohol function, preserved the ?-opioid receptor type-selectivity of parent natural peptides. Because of the slow association-

dissociation kinetics of Dmt[1]-derivatives, there is an ambiguity as far as the extent of their ? receptor type selectivity is concerned.

B.) Determination of receptor constants for ?-opioid receptor agonists in mouse vas deferens.

When studying E/(A) relationships (commonly referred to as dose-response relationships) of agonists in isolated tissues it is done with the understanding that the determined agonist potency is the result of a complex chain of receptor- and tissue-related functions ?160; 165?. It is a long-held wish of pharmacologists to devise methods suitable for determine agonist affinities by pharmacological means for all sets of agonists and also to characterize the agonist property referred to as "intrinsic efficacy" ?43; 167?. Methods for determining the affinity of pure, competitive receptor antagonists are readily available ?8; 73; 144; 166; 175?; it is also feasible to calculate the affinities of a subset of partial agonists ?73?. A method, based on the fractional inactivation of the receptor pool in a given tissue has been devised by Furchgott and Bursztyn ?42? and was used successfully thereafter to establish the affinities and efficacies of a wide range of agonists ?37; 131; 132; 151?.

In the opioid field, the fumaramate derivative of naltrexone, ?-funaltrexamine has been reported as a potentially useful tool for the irreversible inactivation of ?-opioid receptors ?163?. It is an irreversibly inactivating antagonist at ?, to a much lesser extent at ? opioid receptors and also a reversible ?-opioid receptor agonist ?39; 52; 176; 177?. The agent has been used previously to determine receptor constants of a limited number of opioid agonists ?15; 24; 80; 127; 128; 150?.

My aim was to analyze pharmacologically the partial agonist properties of endomorphins, substantiated recently by biochemical means ?3; 61; 81; 154? and also to create a comprehensive receptor constant data set for a number of ?-opioid receptor agonists with particular reference to Tyr-Pro-related agonists.

When I decided to use ?-FNA as a research tool, the choice fell on MVD as the assay system for the following reasons:

1- The MVD has been reported to possess a lower ?-opioid receptor reserve as compared to GPI ?82?. This factor was assumed to facilitate the functional reflection of receptor inactivation.

2- The MVD is known to possess a much lower ? receptor pool than the GPI ?82?; this factor was expected to diminish interference from ?-receptor agonism by ?-FNA.

The delicate issue in MVD is the presence of a highly effective set of ?-opioid receptors. Therefore, comparable data set could be obtained only for those agonists where the extent of ?-opioid receptor selectivity is satisfactorily high. This criterion, with one possible exception, appeared to have been met for the agonists studied.

?-FNA treatment caused a rightward shift, slope reduction and, in some cases, a marked E_{max} reduction in agonist dose-response curves but the extent of these changes was different for the different substes of agonists. After having calculated the receptor constants (K_A and residual receptor fraction "q"), as an indirect validation attempt, we sought correlation between the displacing IC_{50} against ^{3}H-naloxone in the rat brain receptor binding assay in the presence of Na^+ and the K_A values determined by pharmacological means (Rónai et al., submitted). For the purposes of correlation we collected receptor-binding data issued from the same laboratory (Institute of Biochemistry, Biological Research Center, Szeged). With the exception of data pair for ?D-Ser^2?-EM-2 the correlation was excellent; taking into account even the deviant data pair for correlational analysis, the correlation was found significant.

The residual receptor fraction after ?-FNA treatment fell into the 11-18 % range for the majority of agonists. If ?-FNA would have affected uniformly the accessibility of binding sites, the same residual binding capacity range could be expected for all ?-opioid receptor agonists; this, apparently, was not the case. A high residual receptor fraction was calculated for a number of endomorphin derivatives, whereas, apparently, a low residual fraction for ?D-Ser^2?-EM-2.

When the efficacy-related ratios (K_A/IC_{50}) were calculated, setting up a rationale for subdivision of agonists into full- and partial subsets was hindered by the apparently very high efficacy obtained for ?D-Ser^2?-EM-2. Since in the case of this analogue

ambiguity arose even in the context of K_A we decided to extend to the rat vas deferens (Al-Khrasani and Rónai, in preparation) where a highly dominant ?-opioid receptor population is thought to be present ?155? with a very low receptor reserve. In this preparation DAMGO and DAMGA behaved as full agonists whereas ?D-Ser^2?-EM-2 had a very low efficacy. The K_e determined for ?D-Ser^2?-EM-2 in this preparation was rather close to the K_A calculated in MVD. To give full explanation to these phenomena will require more extensive analyses. It appears, however, justified to exclude the K_A/IC_{50} ratio calculated for the peptide in MVD when setting up the subdivision of agonists into full and partial agonist subsets. By using the statistical comparisons of K_A/IC_{50} ratios, the proposed subsets are as follows: DAMGO and its amide, DAMGA are clearly full agonists. This rating is likely to hold also for morphiceptin. ?D-Met^2?-EM-2 and normorphine are also possible full agonists although to decide more firmly their status, further refinement might be required. All the other agonists fall into the partial agonist category.

It is also apparent that subsets may exist even among the partial agonists. The most striking feature is the high residual receptor fraction for a number of partial agonist endomorphin derivatives. It has been suggested that different sets of ?-opioid agonists may use distinct "binding pockets" or binding site combinations on the opioid receptor molecule ?76; 77; 103; 118; 130?. The high residual receptor fraction may indicate that this might be the case also for some endomorphin-derivatives.

THE MODULATORY EFFECTS OF ENDOMORPHINS AND DAMGO ON THE FIELD STIMULATION-INDUCED ^{3}H-NOREPINEPHRINE RELEASE FROM ADULT RAT NUCLEUS TRACTUS SOLITARII-DORSAL MOTOR VAGAL NUCLEUS SLICES

This area of rat lower brainstem has been shown to contain several endogenous opioid peptide sets (enkephalins, dynorphins, ?-endorphin and endomorphins) ?1; 93; 94; 123? as well as all the three major opioid receptor types ?91?.

Arakawa et al. ?7? have reported the inhibition of stimulation-induced norepinephrine release by DAMGO in this preparation.

My results fully confirmed the naloxone-reversible inhibitory effect of DAMGO. EM-1 and EM-2 displayed also inhibitory actions; however, in contrast to DAMGO, the inhibition by endomorphins appeared to level off between 10^{-6}-10^{-5} M concentration. It should be mentioned that none of these opioid agonists was as potent inhibitor as the ? 2-adrenoceptor agonist clonidine. The norepinephrine released from this preparation may originate from local A2 neurons but also from projection neurons of locus coeruleus and rostral ventrolateral medulla ?120; 173?. It is possible all the processes releasing norepinephrine, irrespective of their source, are endowed by inhibitory ? $_2$ autoreceptors whereas only a subset contains ?-opioid receptors.

One of the possible reasons for the flattening of the concentration-response curve of a drug, particularly if the agent is a peptide, is the potential inactivation by tissue enzymes. This possibility appeared to be rather unlikely in the light of recent reports on the remarkable resistance of endomorphins against various peptidases ?169?. A characteristic potential cleaving site in endomorphins is splitting after Pro^2 by dipeptidyl-aminopeptidase IV (DAP-IV, EC 3. 4. 14. 5) ?98; 122; 139?. Therefore, I tested the effect of endomorphins in the presence of enzyme inhibitor diprotin A (Ile-Pro-Ile, ?172?. As expected, the enzyme inhibitor was only marginally effective at lower endomorphin concentration and not at all at 10^{-5}M of peptide concentration. Although further experiments are needed to fully justify the conclusion, there is a probability that the "flattening" of endomorphin action at the higher concentration may be due to the partial agonism also in NTS-DVN slice preparation.

CONCLUSIONS

All endomorphin analogs as well as DAMGA are ?-opioid receptor-selective agonists with exception the derivative with a free C-terminus.

The methylation of Tyr^1 at position 2' and 6' enhanced both the activity and affinity of endomorphins without interfering with the selectivity.

Methylation at the ?-carbon atom accompanied by the introduction of a hydroxyl group into position 3' of aromatic ring of Tyr^1 is apparently unfavorable for the opioid receptors.

Substitution of Pro^2 by D-Ser, D-Met or cycloSer in endomorphin-2 results in analogs with agonist activity comparable to that of the parent peptide whereas substitution by L-Ser or Hyp caused a marked decrease in the agonist activity.

Amidated Phe in position 4 of endomorphin-2 is requirable both for the agonist activity and selectivity of the peptide.

Alcohol function at the C-terminal of natural endomorphins cause only a minor reduction in the agonist activity and preserves the selectivity of parent peptides.

Irreversible partial inactivation of ?-receptor pool revealed that DAMGO, DAMGA and morphiceptin are full agonists whereas EM-1, EM-2, EM-1-ol, EM-2-ol and morphine are partial agonists but ?D-Met?^{2}-EM-2 and normorphine may be a full agonists. It appears that the presence of Pro in position 2 and the aromatic amino acid (Phe) in position 4 are prerequisites of partial agonism.

For some partial agonist endomorphins a significantly higher residual receptor fraction remained available after ß-FNA treatment than either for the morphinan-based agonists, enkephalin-based agonists or another Tyr-Pro – based agonist, morphiceptin. This may be taken as a possible indication that these peptides act, at least in part, at different region(s) of the receptor molecule as compared to the other agonists.

At the central level, electrical field stimulus evoked ^{3}H-NE release from adult rat NTS-DVN revealed that DAMGO is a full agonist but endomorphins might behave differently. Additionally, these results confirm the presence of the functional ?-opioid receptors in this region of CNS.

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RELEVANT PUBLICATIONS

Papers

1. **Al-Khrasani, M.**, Elor, G., Yusuf, A. M., Ronai, A. Z., 2003. The effect of endomorphins on the release of 3H-norepinephrine from rat nucleus tractus solitarii slices. Regul Pept. 111, 97-101.
2. **Al-Khrasani, M.**, Orosz, G., Kocsis, L., Farkas, V., Magyar, A., Lengyel, I., Benyhe, S., Borsodi, A., Ronai, A. Z., 2001. Receptor constants for endomorphin-1 and endomorphin-1-ol indicate differences in efficacy and receptor occupancy. Eur.J Pharmacol 421, 61-67.
3. Lengyel, I., Orosz, G., Biyashev, D., Kocsis, L., **Al-Khrasani, M.**, Ronai, A. Z, Tomboly, C., Furst, Z., Toth, G., Borsodi, A., 2002. Side chain modifications change the binding and agonist properties of endomorphin-2. Biochem. Biophys. Res. Commun. 290, 153-161.
4. Kocsis, L., Benyhe, S., Borsodi, A., Rónai, A. Z, **Al-Khrasani, M.**, Magyar, A., Orosz, G. Endomorphin-related peptide alcohols: structure-activity studies. In: Peptides 2000 (Martinez J, Fehrentz J-A, eds.) EDK, Paris, pp. 823-824, 2001.
5. Rónai, A. Z., Kató, E., **Al-Khrasani, M.,** Hajdú, M., Müllner, K., Elor, G., Gyires, K., Fürst, S., Palkovits, M. Age and Monosodium Glutamate Treatment Cause Changes in the Stimulation-Induced [^{3}H]-Norepinephrine Release From Rat Nucleus Tractus Solitarii-Dorsal Vagal Nucleus Slices. Life Sci., accepted.
6. Tömböly, C., Kövér, K., Péter, A., Tourwé, D., Biyashev, D., Borsodi, A., **Al-Khrasani, M.,** Rónai, A. Z., Tóth, G. Structure-activity study on the Phe side-chain arrangement of endomorphins using conformationally constrained analogues. J. Med. Chem., accepted.

Abstracts

a.) Oral presentations

1. **AL-Khrasani, M.**, Orosz, G., Tóth, G., Benyhe, S., Rónai, A. Z. The in vitro analysis of the opioid properties of potent μ receptor type agonist endomorphins and (D-Ala^2, MePhe^4, Gly^5-ol) –enkephalin (DAMGO) and their analogs. Yemeni Scientifc Research Foundation Science conference 2000, abstract vol., p. 13.
2. **AL-Khrasani, M.**, Kocsis, L., Orosz, G., Tóth, G., Rónai, A. Z. Partial and full agonism in Tyr-Pro-related opioid peptides and non-peptide opioids. YSRF Science conference, Sanaa, Yemen, October 11-13, 2001, abstract vol., p. 97
3. **AL-Khrasani, M.**, Orosz, G., Kocsis, L., Farkas, V., Magyar, A., Benyhe, S., Borsodi, A., Rónai, A. Z. Endomorphins and novel endomorphin analogs: general in vitro pharmacology. In Scientific Bulletin International Multidisciplinary Conference, organized by the North University of Baia Mare, Romania, 4th EDITION, 16-22. May 25-26, 2001
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