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A NOVEL NON-OPIOID BINDING SITE FOR ENDOMORPHIN-1

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Endomorphins are natural amidated opioid tetrapeptides with the following structure: Tyr-Pro-Trp-Phe-NH2 (endomorphin-1), and Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2). Endomorphins interact selectively with the μ-opioid or MOP receptors and exhibit nanomolar or sub-nanomolar receptor binding affinities, therefore they suggested to be endogenous agonists for the μ-opioid receptors. Endomorphins mediate a number of characteristic opioid effects, such as antinociception, however there are several physiological functions in which endomorphins appear to act in a fashion that does not involve binding to and activation of the μ-opioid receptor. Our recent data indicate that a radiolabelled [^{3}H]endomorphin-1 with a specific radioactivity of 2.35 TBq/mmol - prepared by catalytic dehalogenation of the diiodinated peptide precursor in the presence of tritium gas - is able to bind to a second, naloxone insensitive recognition site in rat brain membranes. Binding heterogeneity, i.e., the presence of higher (K_d = 0.4 nM / B_{max} = 120 fmol/mg protein) and lower (K_d = 8.2 nM / B_{max} = 432 fmol/mg protein) affinity binding components is observed both in saturation binding experiments followed by Schatchard analysis, and in equilibrium competition binding studies. The signs of receptor multiplicity, e.g., curvilinear Schatchard plots or biphasic displacement curves are seen only if the non-specific binding is measured in the presence of excess unlabeled endomorphin-1 and not in the presence of excess unlabeled naloxone. The second, lower affinity non-opioid binding site is not recognized by heterocyclic opioid alkaloid ligands, neither agonists such as morphine, nor antagonists such as naloxone. On the contrary, endomorphin-1 is displaced from its lower affinity, higher capacity binding site by several natural neuropeptides, including methionine-enkephalin-Arg-Phe, nociceptin-orphanin FQ, angiotensin and FMRF-amide. This naloxone-insensitive, consequently non-opioid binding site seems to be present in nervous tissues carrying low density or no μ-opioid receptors, such as rodent cerebellum, or brain of μ-opioid receptor deficient ($MOPr^{-/-}$) transgenic or 'knock-out' (K.O.) mice. The newly described non-opioid binding component is not coupled to regulatory G-proteins, nor does it affect adenylyl cyclase enzyme activity. Taken together endomorphin-1 carries opioid and, in addition to non-opioid functions that needs to be taken into account when various effects of endomorphin-1 are evaluated in physiological or pathologic conditions.

Key words: *tritiated endomorphin-1, naloxone insensitive site, μ-opioid peptide receptor, rat brain, radioligand binding, knock-out mice*

INTRODUCTION

It has been almost four decades since the three endogenous opioid peptide families, enkephalins, endorphins and dynorphins, have been identified and characterized (1). Pharmacological approaches have shown the existence of three types of opioid peptide receptors μ (MOP), δ (DOP), and (KOP) (2, 3). Although selectivity of the enkephalins for the δ-receptor (4) and of dynorphins for the -receptor (5) was demonstrated, no specific endogenous ligand had been attributed to the μ-receptor. The pro-opiomelanocortin (POMC) product β-endorphin (6) exhibited very good affinities for each type of opioid receptors, without showing a genuine preference for any of them. About two decades later two endogenous, potent, and selective opioid peptides, named endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), were isolated from bovine brain (7). They produced antinociception in mice after intracerebroventricular administration and displayed extraordinarily high selectivity toward μ-opioid receptors in radioreceptor binding assays. It was concluded therefore that these peptides might be natural ligands for the μ-receptors.

Opioid receptors are coupled to heterotrimeric G-proteins (8) and, upon ligand-activation, are known to inhibit adenylyl cyclase (9). Accordingly, like other μ-receptor specific ligands,

endomorphins stimulate Gi protein activation (10-13), inhibit adenylyl cyclase (10, 11, 14) and induce agonist stimulated receptor internalization (15, 16).

Despite all previous evidence on μ-receptor selectivity of endomorphins some studies do not support the idea of exclusive action of endomorphins through μ-receptors. Thus, endomorphin-1 induced antinociception was not reversed by μ_1-receptor selective antagonist naloxonazine in mice (17), and antagonists beta-funaltrexamine and naloxonazine were ineffective in antagonizing endomorphin-1's antinociceptive effect in diabetic mice (18). The experiments with endomorphin-1 stimulated viral replication in microglial cell culture also suggest that endomorphin-1 acts through 'atypical' μ-receptors (19). Fischer and Undem (20) demonstrated that endomorphins produced a concentration-dependent inhibition of the electrical field stimulation-induced tachykinin-mediated contractions of the guinea pig bronchus. Surprisingly, only endomorphin-1 effects could be blocked by naloxone (10 μM), whereas endomorphin-2 effects were not affected by any opioid receptor-specific antagonist. Endomorphins evoke different cardiorespiratory effects of which ventilatory responses are probably non-opioid-mediated, as they were not attenuated by opioid antagonists (21).

Tachykinin agonist and antagonist pepetides posses a wide range of physiological functions from pain regulations to cancer growing (22). Kosson *et al.* (23) showed weak but significant receptor affinity of endomorphin-1 on NK_1- (70 μM) and NK2- (6.2 μM) receptors and endomorphin-2 for NK_2- (72 μM) receptors. Functional bioassays show weak tachykinin antagonist properties of endomorphin-1 and -2. Although the affinities of endomorphin-1 to NK_2- and NK_1- receptors are 6000 and 70,000 times lower than its affinity for the μ-receptor, this 'tachykinin component' may nonetheless play a significant modulatory role of the major opioid agonist function of endomorphin-1. Botros *et al.* (24) found that endomorphin-2 showed a comparatively high binding affinity (7.5 ± 0.7 nM) for the SP1-7 substance P sites, whereas endomorphin-1 exhibited weak binding (1030 ± 43 nM). This further supports that endomorphin-2 in addition to its affinity for the μ-receptor also may interact with a non-opioid site.

Therefore, we hypothesized that endomorphin-1 may bind to more than one receptor/binding-sites. To study the binding of endomorphins we generated tritiated endomorphin-1 and found that [^{3}H]endomorphin-1 has at least two clearly distinguishable binding sites, one that appears to be a high affinity μ-receptor site while the other site has a somewhat lower affinity but it is fully independent of μ-opioid receptors.

MATERIALS AND METHODS

Chemicals

Tyr-[3,4-$^3H_2$]Pro-Trp-Phe-NH_2 ([^{3}H]endomorphin-1; 2.35 TBq/mmol) was synthesized in our institute (25). Guanosine-5'-[γ-^{35}S]-triphosphate (1204 Ci/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary). Naloxone was from Endo Labs (Garden City, NJ, USA). Morphine and naltrindole were from ICN-Hungary Co. (Tiszavasvari, Hungary). Hemorphin was prepared as described in Szikra *et al.* (26). Angiotensin IV-was from Sigma. Neuropeptide FF (NPFF) was a kind gift from Laszlo Prokai (Department of Molecular Biology and Immunology, University of North Texas, Health Science Center at Fort Worth? Fort Worth, TX, USA). DAMGO [D-Ala^2-(N-Me)Phe^4-Gly^5-ol] Enkephalin, Nociceptin, Leu-enkephalin, Met-enkephalin and β-endorphin were purchased from Bachem (Bubendorf, Switzerland). AMPA, GABA, Glu, (–)MK801 and ()MK801 was from Tocris (Bristol, United Kingdom). SKF (–) and SKF (+), ( ) and (+)N-allylnormetazocine (SKF-10,047) were generous gifts from NIDA (Research Triangle Park, NC, USA). Dextrorphan and levorphanol were purchased from Hoffmann-La Roche (Nutley, NJ, USA). Bovine serum albumin (BSA) and Coomassie Brilliant Blue G250 were from Serva Feinbiochemica GmbH (Heidelberg, Germany). Ready Safe liquid scintillant was purchased from Beckman (Fullertone, CA, USA).

Endomorphin-1, endomorphin-2, H-Tyr-Gly-Gly-Phe-Met-Arg-Phe-OH (MERF) (27), and Ile^{5,6}-deltorphin II (28), dynorphin (1-11), FMRF, and TIPP (H-Tyr-Tic-Phe-Phe-OH) (29) were prepared by solid phase peptide synthesis and purified by high performance liquid chromatography in our institute.

All other chemicals used in this study were of analytical grade and purchased from Sigma (St. Louis, USA) or Reanal/Egis Pharmaceuticals (Budapest, Hungary).

Animals

Three days old and adult male albino Wistar rats with 250 – 280 g body weight (Human Rt., Godollo, Hungary) were used in the study. They were housed in the local animal house of the Biological Research Center (BRC, Szeged, Hungary). Rats were kept in groups of six, allowed free access to food and water and maintained on a standard 12-h light:12-h dark cycle (lights on between 06:00 and 18:00 h) until the time of sacrifice. Animal experiments were performed under the control of the following declaratory statutes: the European Communities Council Directives (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§).

Brains of μ-receptor knockout mice were kindly provided by Professor Brigitte L. Kieffer (UPR 9050 CNRS, ESBS Universite Louis Pasteur, 67400 Illkirch, Strasbourg, France). The generation of μ-receptor deficient mice was described previously (30).

Preparation of rat brain membranes

A crude membrane fraction was prepared from Wistar rat forebrains according to a method of Pasternak with small modifications (31, 32). The animals were decapitated, and the brains without cerebella were rapidly removed, and washed several times with chilled 50 mM Tris-HCl buffer (pH 7.4). The brains were weighed and suspended in 5 (v/w) of brain tissue of the ice-cold buffer. Tissues were homogenized by a Braun teflon-glass homogenizer (10 – 15 strokes, 1000 rev./minute), and filtered through four layers of gauze to remove large aggregates. The volume of the suspension was supplemented to a final buffer volume/membrane pellet ratio of 30 (ml/g). After centrifugation with a Beckman J21M apparatus (40,000 × g, at 4°C, for 20 min, JA20 rotor), the resulting pellet was resuspended in fresh buffer (30 v/w) using a vortex. The suspension was incubated at 37°C for 30 min to remove any endogenous opioids. Centrifugation was repeated under the same conditions as described above, and the final pellet was resuspended in five volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose to give a final protein concentration of 3 – 4 mg/ml. Aliquots (5 ml) of the membrane samples were frozen in liquid nitrogen and then stored at –80°C. The protein concentration was determined by the Bradford method (33) using bovine serum albumin as standard. Before use the membranes were thawed and resuspended in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged (40,000 × g, at 4°C, for 20 min) to remove sucrose and used immediately in binding assays.

Preparation of cerebellar homogenates

Following cervical dislocation, the brains were rapidly removed. Cerebella were dissected and homogenized in 10

volumes of an ice-cold homogenization buffer of the following composition (in mM): TrisHCl pH 8.0, 10; EDTA, 2; DTT, 1; PMSF, 0.5. Homogenates were aliquoted and stored at –80°C until use.

Radioreceptor binding assay

Radioligand binding experiments were performed as described (32). Briefly, the membrane suspensions from rat brain (protein concentration of 0.3 – 0.5 mg/ml) were incubated in glass tubes for 45 min at 24°C with the radioligand in a final volume of 1 ml. Incubations were carried out in 50 mM Tris-HCl buffer (pH 7.4). Incubation was started by the addition of membrane suspension and terminated by rapid filtration through Whatman GF/C glass fiber filters using a Brandel M24R Cell Harvester. After three washings with 5 ml portions of ice-cold buffer (50 mM Tris-HCl, pH 7.4) filters were dried for 3 hours at 37°C. The radioactivity was measured in a toluene-based scintillation cocktail, using a Wallac 1409 scintillation counter. Experiments were carried out in duplicate and repeated several times. Ligand binding data were evaluated by GraphPad Prism 4.0 (34), using a non-linear least-squares algorithm. Data are generally expressed as arithmetic means ± S.E.M of at least three repeated assays.

[^{35}S]GTPγS binding experiments

Functional coupling of the activated receptors with the cell membrane $G_{i/o}$-proteins was measured by [^{35}S]GTPγS binding assays. Rat brain membranes (containing ~10 µg of protein/sample tube) were incubated for 60 min at 30°C in Tris-EGTA buffer (50 mM Tris-HCl buffer, 3 mM $MgCl_2$, 1 mM EGTA, 100 nM NaCl, pH 7.4) containing 0.05 nM [^{35}S]GTPγS with increasing concentrations (10^{-9} 10^{-5} M) of opioid ligands tested in the presence of 30 µM GDP in a final volume of 1 ml. For positive control 10^{-5} M DAMGO was used. Basal activity taken as 100% specific binding was measured by subtracting the non-specific binding (determined in the presence of 100 µM unlabeled GTPγS) from the total binding (measured in the absence of tested compounds). The incubation was started by the addition of [^{35}S]GTPγS and was terminated by rapid vacuum filtration through Whatman GF/B glass fiber filters. Filters were washed three times with ice-cold Tris-HCl buffer (50 mM, pH 7.4) and then dried. The bound radioactivity was measured in a ready safe scintillation cocktail, using a Wallac 1409 liquid scintillation spectrometer. G-protein stimulation is given as percentage of the basal activity. Data were calculated from three independent experiments performed in triplicates. Efficacy (E_{max}) and potency (EC_{50}) values Data were calculated by fitting sigmoid dose-response curves using the GraphPad Prism 4.0 program (34).

Measurement of adenylyl cyclase activity

Adenylyl cyclase assay was performed on brain homogenates of rat cerebellum and cortex. Brain homogenates were pre-incubated with the agonists or with vehicle for 10 min at room temperature. When used, antagonist was added 10 min before adding an agonist. Reaction mixtures contained 15 – 40 µg protein, 50 mM Tris-HCl, 5 mM $MgCl_2$, 10 µM GTP, 10 mM cAMP (containing 20,000 cpm ^{3}H-cAMP; Amersham, France), 1 mM ATP (containing 106 cpm [α-^{32}P]-ATP; Amersham, France), 5 mM creatine phosphate and 250 µg/ml creatine kinase in a total volume of 60 µl. After 10 min of incubation at 35°C, reactions were stopped with 500 mM HCl then neutralized with 1.5 M imidazole. The assay mixture was then loaded on alumina columns to separate cAMP from ATP. Column flow-through was measured by liquid scintillation counting using two counting windows for ^{3}H and ^{32}P, to determine the amount of [^{3}H]cAMP and [^{32}P]cAMP, respectively. The formed [^{32}P]cAMP amount was then corrected for the recovery of the added [^{3}H]cAMP. All measurements were run in triplicates. Protein amount was determined using the BioRad protein assay (BioRad, Germany). Data were analysed with Prism 4.0 from GraphPad (34).

RESULTS

[^{3}H]endomorphin-1 was prepared by dehalotritiation method through [3',5' -diI-Tyr^1]endomorphin-1, which was tritiated in the presence of Pd/$BaSO_4$. The final product had a specific radioactivity of 2.35 TBq/mmol (25). The specific binding of [^{3}H]endomorphin-1 was temperature-dependent (data not shown) and the highest specific binding was found to be at 24°C using glass test tubes and C type of Whatmann glass fiber filter. Addition of different enzyme inhibitors to experimental buffer had no positive effect on specific binding; therefore, all the following experiments were performed using 50 mM Tris buffer (pH 7.4). Kinetic studies revealed that [^{3}H]endomorphin-1 binding reaches the steady state level in 45 minutes of incubation (data not shown). These conditions were used throughout the following experiments.

Saturation binding experiments were carried out in rat brain membranes at varying concentrations of the radiolabelled endomorphin-1 (from 0.01 nM to 100 nM) and non-specific binding was determined either in the presence of 10^{-5} M naloxone (*Fig. 1A*, open circles) or 10^{-5} M unlabelled endomorphin-1 (*Fig. 1A*, filled circles). Specific binding was found to be saturable in both cases. Surprisingly, we found that specific binding of [^{3}H] endomorphin-1 with nonspecific binding measured by unlabelled endomorphin-1 was significantly higher than [^{3}H]endomorphin-1's specific binding with nonspecific binding determined in the presence of unlabelled naloxone (*Fig. 1A*).

The equilibrium dissociation constant (K_d) and the maximal number of binding sites (B_{max}) were calculated by non-linear regression analysis. The obtained results indicated the existence of a single binding site when 10^{-5} M naloxone was used to determine non-specific binding (K_d of 1.0 ± 0.1 nM and the B_{max} was 188.0 ± 21.7 fmol/mg protein). Linear regression analysis of the data after Scatchard transformation confirmed the existence of a single binding site with naloxone (*Fig. 1B*).

After determining the non-specific binding by using 10^{-5} M endomorphin-1 the nonlinear regression analysis showed that the best fit could be obtained by an equation modelling two binding sites. The equilibrium dissociation constants were K_{d1}= 0.4 ± 0.1 nM and K_{d2}= 8.2 ± 1.4 nM and the corresponding B_{max} values were 119.6 ± 18.5 and 432.5 ± 83.7 fmol/mg protein, respectively. The Scatchard transformation of the binding isotherm was curvilinear, also suggesting the existence of two classes of binding sites (*Fig. 1C*). Non-specific binding of [^{3}H]endomorphin-1 was under 30% of total binding at a radioligand concentration of 0.4 nM which increased to 60% at 8.2 nM (data not shown). Both the high and low affinity binding of [^{3}H]endomorphin-1 to rat brain membranes were blocked by heat treatment (50°C for 60 min at 8.5 nM [^{3}H]endomorphin-1 radioligand concentration) (*Fig. 2*) or by N-ethyl-maleimide pre-treatment or by α-chymotrypsin pre-treatment (data not shown) suggesting that both the high and low affinity [^{3}H]endomorphin-1 binding sites are composed of proteins.

The existence of two binding sites for endomorphin-1 was further confirmed by equilibrium homologous competition experiments resulting in a biphasic curve (*Fig. 3*). [^{3}H]endomorphin-1 (0.4 nM) was displaced by endomorphin-1

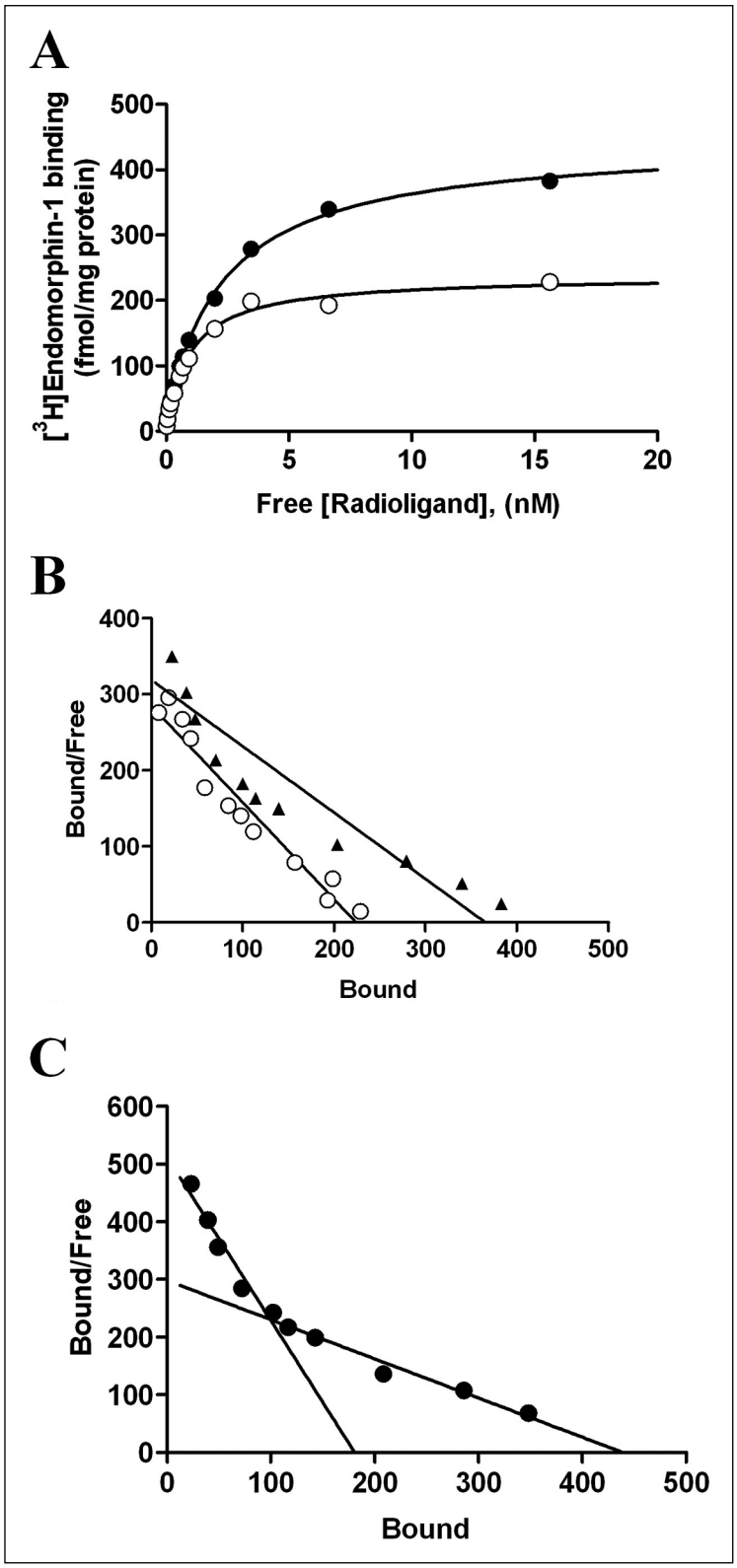

Fig. 1. Saturation binding curve and Scatchard plot of [^{3}H]endomorphin-1 binding.
(A) Summary of saturation binding isotherms for [^{3}H]endomorphin-1. (B) Scatchard plot of [^{3}H]endomorphin-1 binding, using 10^{-5} M naloxone to determine non-specific binding. (C) Scatchard plot of [^{3}H]endomorphin-1 binding, using 10^{-5} M endomorphin-1 to determine non-specific binding. Rat brain membranes were incubated with increasing concentration of [^{3}H]endomorphin-1 in the presence and absence of 10^{-5} M naloxone (○) or 10-5 M endomorphin-1 (●) for 45 min at 24°C. Graphs depict a representative experiment, carried out in duplicates and repeated two times with similar results.

in a concentration dependent manner giving an interim plateau at around 35% of total binding. This higher affinity binding had a K_d value in the subnanomolar concentration range (K_{d1} = 7.2 ± 3.0 pM). The remaining ~35% of [^{3}H]endomorphin-1 binding was displaced by a K_d value that was more than one order of magnitude lower (K_{d2} = 8.9 ± 1.5 nM). These higher and lower affinity values were comparable to those obtained from saturation binding experiments.

The high affinity endomorphin-1 binding (0.4 nM) was displaced by MOP receptor ligands with high affinity (K_i in the nanomolar range) and by DOP or KOP ligands with much lower affinity (*Table 1*). The binding of endomorphin-1 to μ-receptor was stereospecific, demonstrated by the displacement of [^{3}H]endomorphin-1 with levorphanol (K_i value of 0.31 ± 0.13 nM) and its pharmacologically inactive enantiomer dextrorphan (K_i > 400 nM) (*Table 1*). The marked differences (three orders

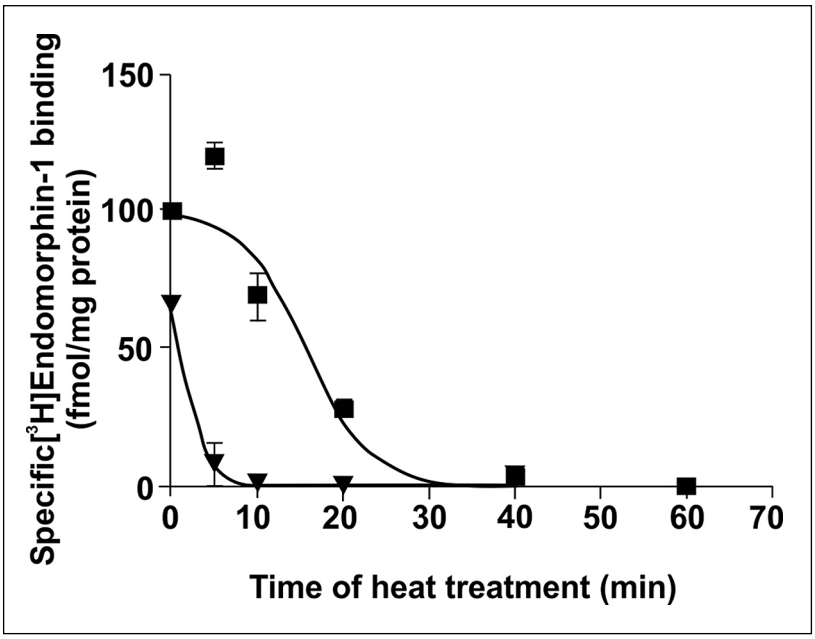

Fig. 2. Effect of heat denaturation on [^{3}H]endomorphin-1 binding. Rat brain membranes were incubated for various time intervals at 50°C with 8.5 nM [^{3}H]endomorphin-1 in the presence and absence of 10^{-5} M naloxone (▼) or 10^{-5} M endomorphin-1 (■). Points represent means ± S.E.M. of at least three experiments, carried out in duplicates.

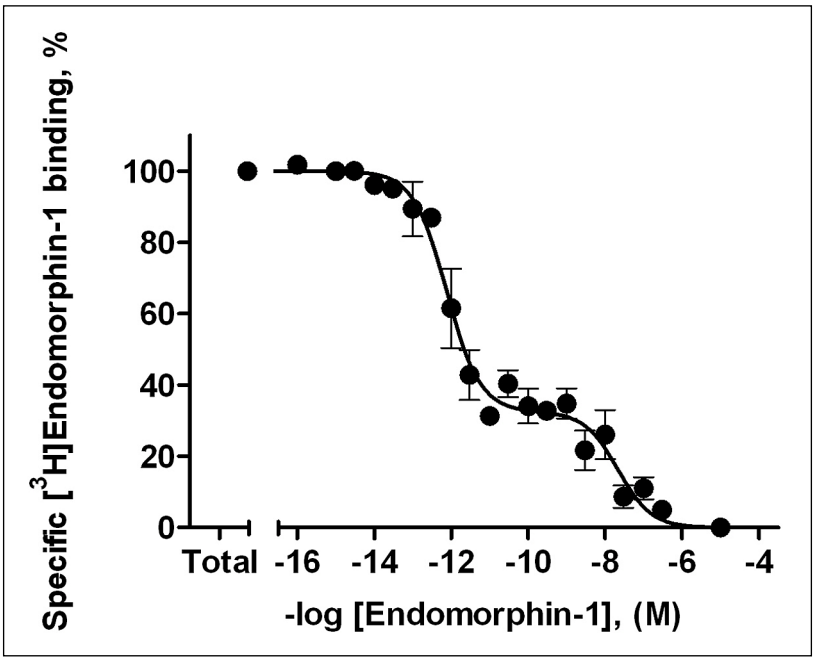

Fig. 3. Homologous displacement of [^{3}H]endomorphin-1 on rat brain membrane. Rat brain membranes were incubated with 0.4 nM [^{3}H]endomorphin-1 for 45 min at 24°C in the presence of increasing concentrations (10^{-16} – 10^{-5} M) of endomorphin-1. Data were analyzed by both one-site and two-site competition models, and they were compared with an F test to obtain optimal fit. The curve represents the result of the two site fits. Specific binding was determined by subtracting the values obtained in the presence of 10^{-5} M endomorphin-1 from the total binding and used for the normalization of the data as 100%. Data points are the averages of at least 3 independent experiments in duplicates ± S.E.M.

of magnitude) in the equilibrium inhibition constant values of the stereoisomer compounds reflect stereoselective site recognition (35). These features suggest that the high affinity binding occurs on μ-receptors. The binding selectivity of [^{3}H]endomorphin-1 towards mu binding site was about 1000 fold higher than for delta site, and about 20 fold higher, than for kappa binding site.

To determine the receptor specificity of the naloxone insensitive low affinity binding site for endomorphin-1, heterologous displacement experiments were carried out in the presence of 8 nM [^{3}H]endomorphin-1 and a wide range of opiate and non-opioid receptor ligands at a fixed 10^{-5} M concentration in the presence of 10^{-5} M naloxone (*Fig. 4*). Based on the data we obtained the results can be categorized into several groups: i) ligands that had no effects on [^{3}H]endomorphin-1 binding in the absence or presence of naloxone (these were some of the ligands for glutamate, GABA, sigma, dopamine, nor-epinephrine and serotonin receptors); ii) ligands that partially displaced [^{3}H]endomorphin-1 binding in the absence of naloxone to a lesser extent than naloxone itself, but in the presence of naloxone they had no effect on [^{3}H]endomorphin-1 binding; iii) ligands that displaced as much [^{3}H]endomorphin-1 as naloxone, but in the presence of naloxone they had no additional effect (DAMGO, morphine, naltrindole or Ile ^{5,6}deltorphin, SKF10,047(–)); iv) ligands that were at least as potent as naloxone itself in displacing [^{3}H]endomorphin-1, and addition of naloxone produced further, but not complete, [^{3}H]endomorphin-1 displacement; v) ligands that fully displaced [^{3}H]endomorphin-1 binding, consequently the addition of naloxone had no further effect, like: endomorphin-1, endomorphin-2, β-endorphin, dynorphin(1-11), methionine-enkephalin, nociceptin and MERF; vi) ligands that displaced only 35 – 40% [^{3}H]endomorphin-1 binding themselves, however, in

Table 1. Potencies of various opioid ligands in inhibiting 0.4 nM [^{3}H]endomorphin-1 binding to rat brain membranes.

Selectivity	Ligand	K_i (nM) ± S.E.M.
General opioid antagonist	Naloxone	0.62 ± 0.07
μ-receptor selective	Endomorphin-1	1.00 ± 0.1
	Endomorphin-2	2.23 ± 0.08
	DAMGO	0.84 ± 0.06
δ-receptor selective	TIPP (Tyr-Tic-Phe-Phe)	739.33 ± 51.67
	Ile^{5,6}- Deltorphin-II	1110.67 ± 85.76
κ-receptor selective	Dynorphin(1-17)	15.05 ± 3.73
	MERF	20.06 ± 6.35
Opioid agonist, active stereomer	Levorphanol	0.31 ± 0.13
Opioid agonist, inactive stereomer	Dextrorphan	481.67 ± 81.53

Rat brain membranes were incubated for 45 min at 24°C with 0.4nM [^{3}H]endomorphin-1 in the presence of increasing concentrations of opioid ligands. K_i values were calculated by fitting displacement binding curves using Graphpad Prism 4.0 program non-linear least-squares algorithm. Values represent the means ± S.E.M. of several independent experiments performed in duplicate.

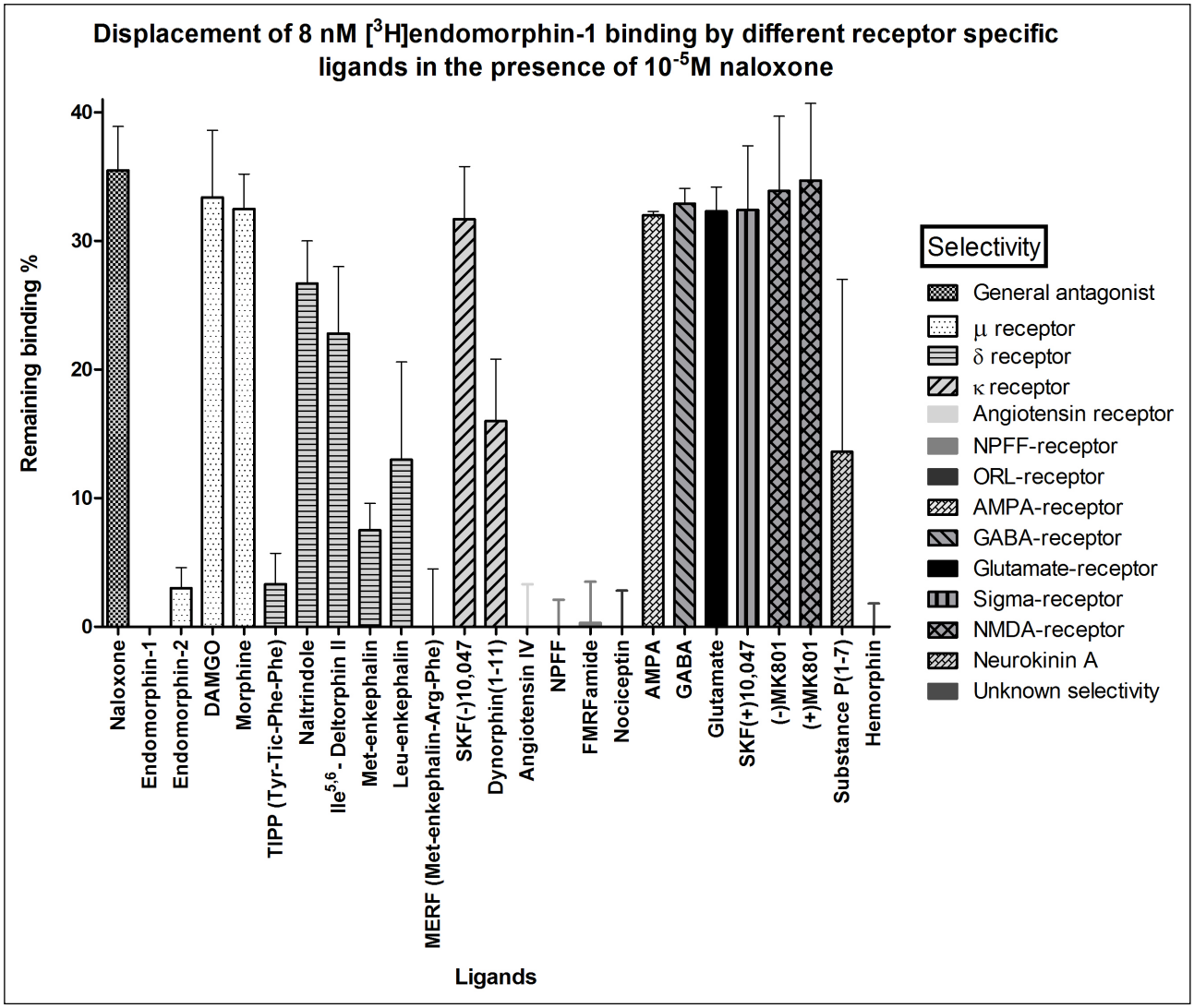

Fig. 4. Displacement of 8 nM [^{3}H]endomorphin-1 binding by different receptor specific ligands in the presence of 10^{-5}M naloxone. Heterologous displacement experiments were carried out in rat brain membranes incubated at 24°C for 45 min in the presence of 8 nM [^{3}H]endomorphin-1 and a wide range of opiate and non-opiate receptor ligands at a fixed 10^{-5} M concentration in the absence or presence of 10^{-5}M naloxone. Values represent the means ± S.E.M. of three independent experiments performed in duplicate.

the presence of naloxone, the [^{3}H]endomorphin-1 binding was fully displaced. Peptides in this last group (angiotensin IV, FMRF-amide, NPFF) appear to bind exclusively to the naloxone insensitive binding site of endomorphin-1.

Following the basic characterization of the high and low affinity binding sites for [^{3}H]endomorphin-1 in rat forebrain we checked, whether the low affinity binding occurs in tissues that have no or very few opioid receptors. Therefore, we prepared crude

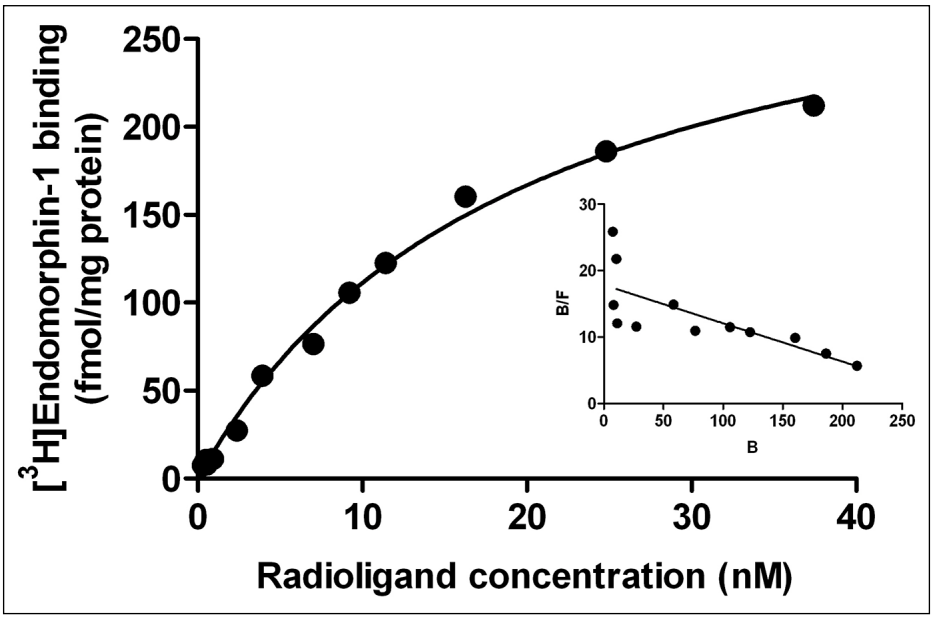

Fig. 5. Saturation binding isotherm for [^{3}H]endomorphin-1 in cerebellar homogenate. Rat cerebellar homogenates were incubated with increasing concentration of [^{3}H]endomorphin-1 in the presence and absence of 10^{-5} M endomorphin-1 (●) for 45 min at 24°C. Each value represents the mean of a single experiment, carried out in duplicates and repeated two times with similar results.

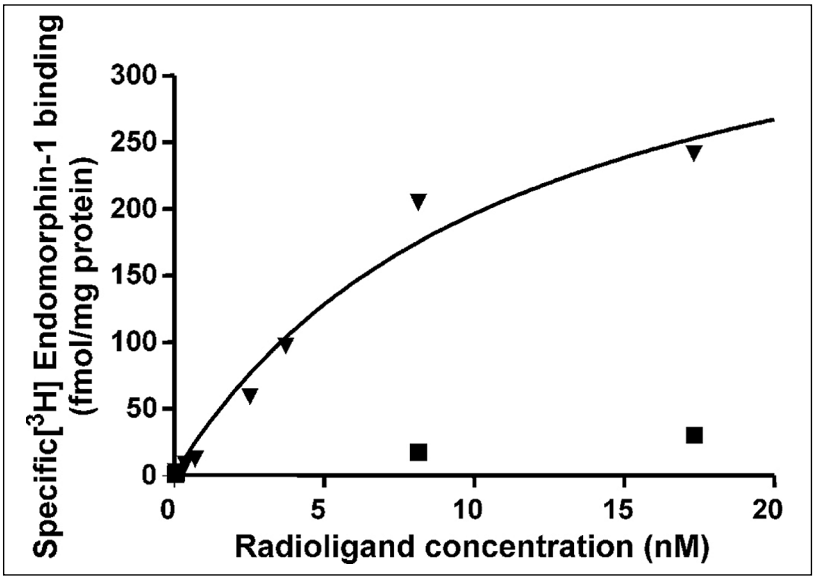

Fig. 6. Saturation binding isotherm for [^{3}H]endomorphin-1 in brains of μ-opioid receptor KO mice. Brain membranes of μ-receptor KO mice were incubated with increasing concentration of [^{3}H]endomorphin-1 in the presence and absence of 10^{-5} M naloxone (■) or 10^{-5} M endomorphin-1 (▼) for 45 min at 24°C. Each value represents the mean of a single experiment, carried out in duplicates and repeated two times with similar results.

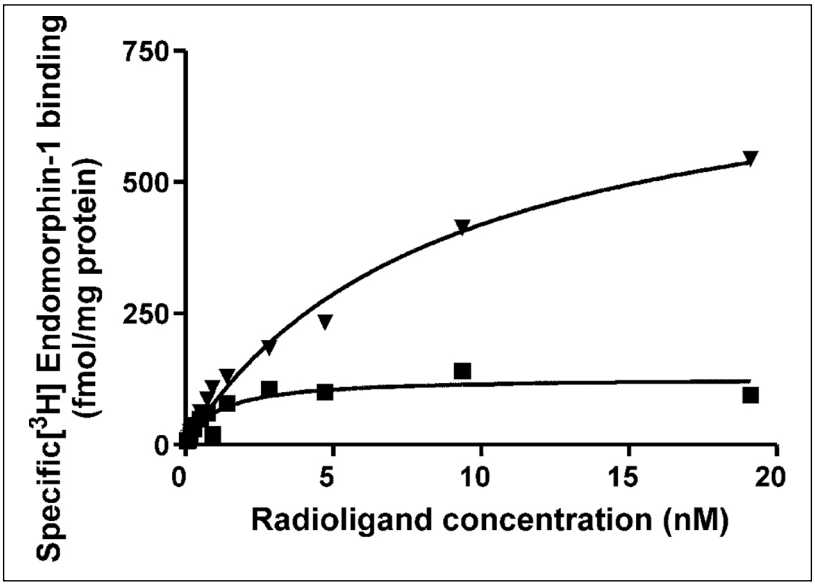

Fig. 7. Saturation binding isotherms for [^{3}H]endomorphin-1 in p3 rat forebrain membrane. P3 rat brain membranes were incubated with increasing concentration of [^{3}H]endomorphin-1 in the presence and absence of 10^{-5} M naloxone (■) or 10^{-5} M endomorphin-1 (▼) for 45 min at 24°C. Each value represents the mean of a single experiment, carried out in duplicates and repeated three times with similar results.

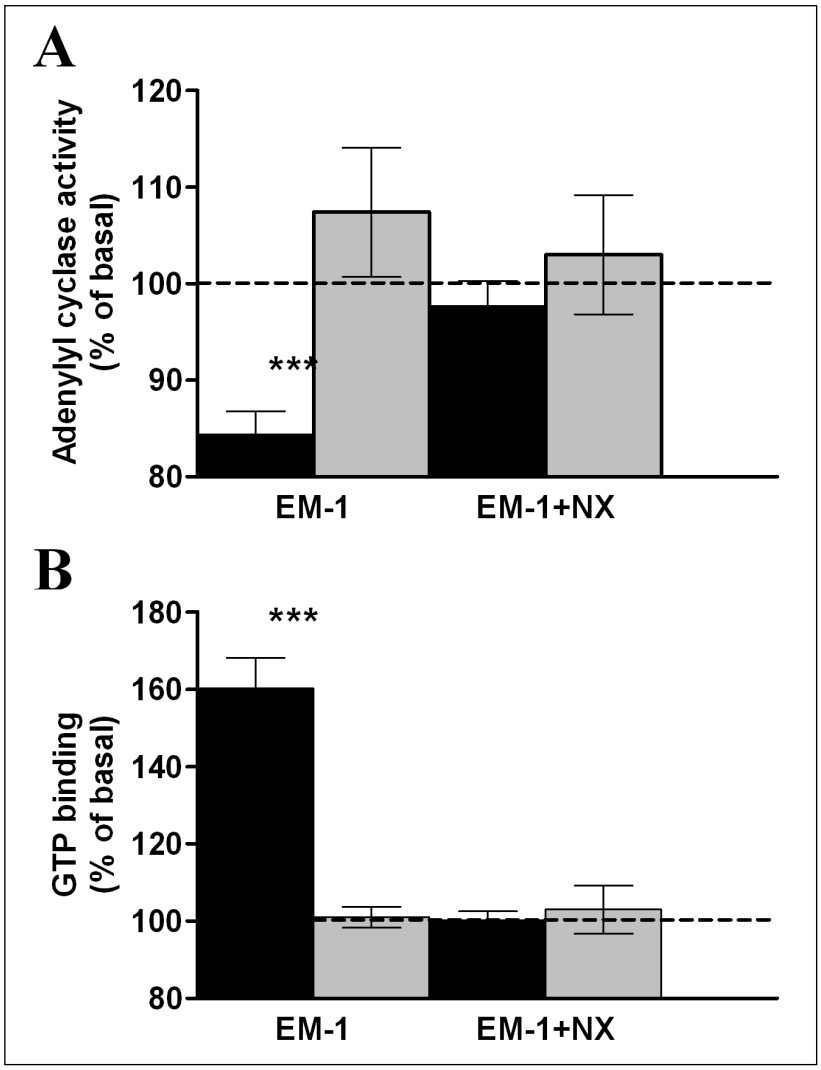

Fig. 8. Effects of endomorphin-1 on signaling. (A) Stimulation of [^{35}S]GTPγS binding by endomorphin-1 in rat forebrain- and cerebellar homogenates. Endomorphin-1 (10^{-5} M) was incubated in the presence or in the absence of opioid antagonists naloxone (10^{-5} M) for 60 min at 30°C as described in Materials and methods. Dark bars represent rat forebrain and light bars represent rat cerebellar samples. Data represent means ± S.E.M. of three to four independent experiments, each performed in triplicate. (B) Inhibition of adenylyl cyclase activity by endomorphin-1 in rat forebrain- and cerebellum, in the presence and absence of naloxone. Adenylyl cyclase activity was measured in rat forebrain- and cerebellar homogenates in the presence endomorphin-1 (10^{-5} M) or endomorphin-1 (10^{-5} M) + naloxone (10^{-5} M). Basal activity (100%) refers to the amount of cAMP generated in the absence of opioid agonist. Data are means ± S.E.M. values from three independent experiments, each performed in triplicate.

homogenates from rat cerebellum that has been shown to be mostly free of μ-receptors (36, 37) and forebrain of μ-receptor K.O. mice (30). As expected, no displacement of [^{3}H]endomorphin-1 binding was observed in the presence of naloxone despite the [^{3}H]endomorphin-1 binding to both membrane preparations (*Figs. 5* and *6*). The binding to rat brain cerebellar membrane was saturable, and best fitted with the single binding site model, giving a K_d of 18.2 ± 2.82 nM and a B_{max} of 403 ± 48.6 fmol/mg (*Fig. 5*). Similarly, in μ-receptor K.O. mouse brains the [^{3}H]endomorphin-1 binding was saturable, fully independent of naloxone giving a K_d of 14.5 ± 1.3 nM and a B_{max} of 423.02 ± 79.5 fmol/mg (*Fig. 6*). Scatchard transformation of both sets of data supported a single binding site model (data not shown).

We tested extracts of three-day old p3 rat brains for endomorphin-1 binding. Interestingly, we found that the proportion of the non-opioid binding site is very high in brains of p3 rats (85 – 90% of the total binding, K_d = 8.2 ± 2.4 nM; B_{max} = 841 ± 42 fmol/mg when endomorphin-1 was used for non-specific binding, K_d = 1.2 ± 0.2 nM; B_{max} = 127 ± 17 fmol/mg when naloxone was used for non-specific binding.), suggesting that this low affinity, non-opioid [^{3}H]endomorphin-1 binding site is present in rodent brain from an early age (*Fig. 7*).

Next we examined whether the signalling pathway activated by μ-receptor is modulated by the low affinity, naloxone insensitive endomorphin-1 binding site. The stimulation of GTP-binding and the inhibition of adenylyl cyclase activity were measured in rat cortical and compared to cerebellar membranes that served as a negative control. Endomorphin-1 produced a 60% increase in [^{35}S]GTPγS binding and a 15% decrease in adenylyl cyclase activity in rat cortical membranes compared to basal values (*Fig. 8A* and *8B*). However, both of these changes were fully reversed by the addition of 10^{-5} M naloxone. In addition, endomorphin-1 did not affect [^{35}S]GTPγS binding and adenylyl cyclase activity in membranes prepared from rat cerebellum. These results suggest that the naloxone insensitive binding does not contribute to the classical μ-receptor mediated signalling events.

DISCUSSION

The discovery of endomorphin-1 and -2 by Zadina *et al.* (7) was a major breakthrough in opioid research. These two peptides are thought to be natural ligands for the μ-opioid receptors. In spite of the vigorous research for about three decades after the description of the endogenous ligands for the δ- and κ-receptors (enkephalins and dynorphins) (2, 5), there was no acceptable candidate for the μ-receptors. Opioid receptors participate a wide range of physiological functions incuding pain regulations (38) and gastrointestinal transit (39). Endogenous opioid peptides are as good painkillers as opiate alkaloids, although systematically given peptides have to be protected against rapid inactivation by peptidase enzymes. A highly potent inhibitor of such peptide degrading enzymes has recently been introduced (40).

Since the discovery of the endomorphins in 1997, several studies were undertaken for a detailed pharmacological characterization of these compounds (41, 42). Endomorphin-1 and 2 was prepared in tritiated form (25, 43) for a more complete characterization of these peptides (10, 11).

In the present paper we report a comprehensive study including binding properties of [^{3}H]endomorphin-1 and found that endomorphin-1 has at least two clearly distinguishable binding sites, one that appears to be the μ-receptor site while the other site has a somewhat lower affinity but it is fully independent of μ-opioid receptors. Non-opioid effects have already been described for other opioid peptides (for review see 27). Therefore it is not unprecedented that endomorphins can also bind to non-opioid binding sites. There is plenty of evidence suggest that this can happen, as endomorphins had been shown to be involved in μ-receptor independent antinociception (17, 18), viral replication (19), tachykinin-mediated contractions of the guinea pig bronchus (20), excitatory ventilatory responses (21), binding to the neurokinin receptors (23) as demonstrated by subtance P (1-7) fragment (24). It also seems that the endomorphin-2 binding to the substance P fragment site could be blocked by naloxone (24).

In radioligand binding assays (RBA) the level of non-specific binding has to be determined in the presence of excess (around thousand fold concentration) unlabelled receptor ligand. Generally two types of such measurements exist: the non-specific agent is chemically same as the radioligand (homologous competition, e.g., nanomolar [^{3}H]endomorphin-1 versus micromolar unlabelled endomorphin-1); the chemical structure of the radioprobe and the non-specific ligand is different (heterologous competition, e.g., nanomolar [3H]endomorphin-1 versus micromolar unlabelled naloxone). In theory both method helps in determining specific ligand binding giving the same or very similar numeric values. However, in our practice naloxone and endomorphin-1 gave rather different results in calculating the level of non-specific binding. Naloxone produced monophasic competition curves (not shown), while using endomorphin-1 as non-specific compound clearly biphasic displacement curves composed of higher and lower affinity binding components were obtained (*Fig. 3*). The higher affinity portion corresponds to the μ-opioid receptor binding site, which is a typical GPCR recognition site, whereas the remaining binding component represent a neuropeptide-sensitive (enkephalin, nociceptin, NPFF, FMRF-amide, *etc.* accessible, see *Fig. 4*) additional non-opioid binding site. The exact nature of the non-opioid site needs to be determined. This observation of non-opioid binding sites does not stand alone in the literature. Benyhe *et al.* (32) described very similar binding profile for the heptapeptide enkephalin (Met-enkephalin-Arg-Phe, MERF) peptide pair [^{3}H]MERF versus unlabelled MERF in rat brain membranes. The non-opioid binding site for [^{3}H]MERF was further characterised in cerebellar membranes of guinea-pig and rat (35) and suggested to be associated with the sigma2 binding or receptor site. A very similar non-opioid recognition site was described for the hemoglobin originated heptapeptide VV-hemorphin-7 in rat brain membranes (26). The significance of the non-opioid binding sites and non-opiate effects of a number of endogenous opioid peptides was described by Wollemann and Benyhe (27).

Saturation binding experiments were carried out on rat brain membranes. The obtained results indicated the existence of a single binding site when 10^{-5} M naloxone was used to determine non-specific binding (K_d of 1.0 ± 0.1 nM and the B_{max} was 188.0 ± 21.7 fmol/mg protein). Linear regression analysis of the data after Scatchard transformation confirmed the existence of a single binding site with naloxone (*Fig. 1B*). After determining the non-specific binding by using 10^{-5} M endomorphin-1 the nonlinear regression analysis showed that the best fit could be obtained by an equation modelling two binding sites. The equilibrium dissociation constants were K_{d1} = 0.4 ± 0.1 nM and K_{d2} = 8.2 ± 1.4 nM and the corresponding B_{max} values were 119.6 ± 18.5 and 432.5 ± 83.7 fmol/mg protein, respectively. Analysis of the data after Scatchard transformation confirmed that there are two binding sites for endomorphin-1 on rat brain membranes (*Fig. 1C*).

The obtained Bmax value for the high affinity binding of [^{3}H]endomorphin-1 was in agreement with the values reported for μ-receptor density for other μ-receptor selective peptide radioligands, such as [^{3}H]endomorphin-2 (10), [^{3}H]TAPP (40), [^{3}H]PL-017 (41), and [^{3}H]dermorphin (46).

It is important to note that the endomorphin-1 binding appears to be associated with proteins and are not the result of a nonspecific chemical binding as both the high and low affinity bindings were abolished by heat or (*Fig. 2*) or N-ethyl-maleimide and alpha-chymotrypsin (data not shown) pre-treatment.

In the homologous competition experiments (*Fig. 3*) [^{3}H]endomorphin-1 (0.4 nM) was displaced by endomorphin-1 in a concentration dependent manner giving an interim plateau at around 35% of total binding. This high affinity binding had a (K_{d1} = 7.2 ± 3.0 pM), the remaining ~35% of [^{3}H]endomorphin-1 binding was displaced by a K_{d2} = 8.9 ± 1.5 nM. The existence of two binding sites for endomorphin-1 was further confirmed by these homologous displacement experiments. These high and low affinity values were comparable to those obtained from saturation binding experiments. The high affinity endomorphin-1 (0.4 nM) binding was stereospecific as found by displacements of [^{3}H]endomorphin-1 with the biologically potent opioid levorphanol (Ki value of 0.31 ± 0.13 nM) and its pharmacologically inactive enantiomer dextrorphan (Ki > 400 nM) (*Table 1*). The high affinity endomorphin-1 binding (0.4 nM) was displaced by μ-receptor ligands with high affinity (K_i in the nanomolar range) and by δ-receptor or κ-receptor ligands with much lower affinity (*Table 1*). These features suggest that the high affinity binding occurs on the μ-receptors.

Equilibrium binding of [^{3}H]endomorphin-1 was further studied in tissues not expressing opioid receptors. The binding to rat brain cerebellar membrane was saturable, and best fitted with the single binding site model, giving a K_d of 18.2 ± 2.82 nM and a B_{max} of 403 ± 48.6 fmol/mg (*Fig. 4*). Similarly, in μ-opioid receptor KO mice brain (*Fig. 5*) the [^{3}H]endomorphin-1 binding was saturable, naloxone insensitive with a K_d of 14.5 ± 1.3 nM and a B_{max} of 423.02 ± 79.5 fmol/mg.

It has been shown that brains from newborn rats contain very low levels of μ-receptor (47). Accordingly, we found that the proportion of the non-opioid binding site is very high in 3 days old rats (85 – 90% of the total binding, K_d = 8.2 ± 2.4 nM; B_{max} 841 ± 42 fmol/mg when endomorphin-1 was used for non-speceific binding, K_d = 1.2 ± 0.2 nM; B_{max} = 127 ± 17 fmol/mg when naloxone was used for non-speceific binding.), suggesting that this low affinity, non-opioid [^{3}H]endomorphin-1 binding site is present in brain from an early age (*Fig. 6*).

Functional assays were performed on rat cortical- and cerebellar membranes. Endomorphin-1 produced 60% increase in [^{35}S]GTPγS binding and a 15% decrease in AC activity in rat cortical membranes compared to basal values (*Fig. 7A* and *7B*). The maximal inhibition values for the inhibition of adenylyl cyclase were almost identical to that of morphine (10) and they are in good agreement with those reported in the literature (48). However, both of these changes were fully reversed by the addition of 10^{-5} M naloxone. In addition, endomorphin-1 did not affect AC activity and GTP binding in membranes prepared from rat cerebellum. These results suggest that the naloxone insensitive binding does not contribute to the classical μ-opioid receptor mediated signalling events.

Experiments with truncated μ-opioid receptors showed that endomorphin-1 binds to μ-receptor in different manner than other

μ-receptor specific ligands as morphine or DAMGO, or than the non-selective opioid agonist naloxone (49). Competition binding experiments of radio iodinated endomorphin-1 in mouse brain with some endogenous opioid peptides as well as non-peptide opioid ligands (50) demonstrated shallow competition curves with the Hill coefficients far less than unity, suggesting the existence of more than one class of binding sites.

Binding site heterogeneity is well established for the opioid receptors. Early studies showed the presence of high affinity binding component in different membrane preparations (51). This might be the results of allosteric modulation, e.g., by sodium ions. The idea of sodium ions altering G-protein-coupled receptor (GPCR) ligand binding and signaling was first suggested for opioid receptors in the 1970s and subsequently extended to other GPCRs. Whether sodium accesses different receptor subtypes from the extra- or intracellular sides, following similar or different pathways, is still an open question. Earlier experiments in brain homogenates suggested a differential sodium regulation of ligand binding to the three major opioid receptor subtypes, in spite of their high degree of sequence similarity (52). Another reason for the binding heterogeneity might be the different affinities of G-protein coupled and uncoupled receptors. Multiple affinity states and guanyl-nucleotide (GppNHp) sensitivity of the radioligand binding were reported in NG-108,15 neuroblastoma-glioma hybrid cell membranes (53). It was also demonstrated that rates of dissociation of [^{3}H-D-Ala^{2}-D-Leu^{5}]enkephalin from bovine hippocampal synaptic plasma membranes varied depending upon association time, suggesting a multistep binding process. A slowly formed high affinity state appeared to be rapidly converted to a lower affinity state by the addition of GppNHp (54). The presence of naloxone-insensitive binding sites for certain opioid peptides was reported e.g., by Benyhe *et al.* (32) and extensively reviewed by Wollemann and Benyhe (27). The naloxone insensitive site is accessible for a number of endogenous peptide ligands (as shown in *Fig. 4*), while many synthetic ligands and alkaloid compounds are not able to interact with such binding site. Last but not least receptor homo- and hetero-oligomerization might be a further reason of having multiple binding sites. One mechanism for such findings was the generation of novel signaling complexes by receptor hetero-oligomerization, which resulted in significantly different pharmacology for mu- and delta-receptor hetero-oligomers compared with the individual receptors (55). Hetero-dimerization of μ-receptors may not only be studied in heterologous expression systems, but can directly be visualized in living cells (56).

Thus, naloxone insensitive portion of endomorphin-1 binding could be displaced by endogenous peptides with different specificity, as well as synthetic delta compound TIPP, which has strong structural resemblance to endomorphin-1. These experiments proved the existence non-opioid binding of endomorphin-1 in rodent brain. Overall the main conclusion of this work is that we proved the presence of a non-opioid binding site for endomorphins that appears to be responsible for all the physiological actions that had been unrelated to an action through μ-receptor. How these will benefit treatment strategies in the future will require further investigations. However these results highlight the importance for further research on uncovering the whole spectrum of effects of these endogenous peptides.

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