APPENDIX

I

Cs. Tömböly, R. Dixit, I. Lengyel, A. Borsodi and G. Tóth

"Preparation of Specifically Tritiated Endomorphins."

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Preparation of Specifically Tritiated Endomorphins

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SUMMARY

Endomorphin-1 (EM1, Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM2, Tyr-Pro-Phe-Phe-NH_2) are natural tetrapeptide ligands of μ-opioid receptors involved in the modulation and attenuation of pain. For a detailed examination of their receptor-binding properties and their metabolic stability, tritium-labelled EM1 and EM2 radioisotopomers were synthesized by catalytic dehalogenation or saturation of the precursor peptides with tritium gas. Amino acid analysis revealed that the tritium labelling was specific and the specifically labelled radioligands possessed high specific activity, ranging from 0.77 TBq/mmol to 2.35 TBq/mmol. The biological half-lives of the peptides in the biological matrix (295 min and 230 min for EM1 and EM2, respectively) indicate that these radioligands are appropriate for binding assays in rat brain membrane preparations. The radioisotopomers of EM2 are not statistically different in the receptor-ligand interaction, and they are excellent tools for further comparative biochemical studies.

Keywords: endogenous opioid peptides, endomorphins, tritium, specific labelling, radioisotopomers, metabolism

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INTRODUCTION

The main classes of opioid receptors (μ, δ and κ), which differ in their ligand selectivity and anatomical distribution, are present in the nervous system [1] and peripheral tissues [2] of various mammalian species. These receptors and their endogenous ligands, the enkephalins [3], endorphins [4], dynorphins [5] and endomorphins [6], are involved in the modulation and attenuation of pain. Endomorphin-1 (EM1, Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM2, Tyr-Pro-Phe-Phe-NH_2) isolated from the bovine and human brain cortex [6,7] produce antinociception with similar potency to that of morphine [8-10]. [^{125}I]EM binding has been characterized on mouse brain membranes and on the recently cloned μ_1-opioid receptor stably expressed in Chinese hamster ovary cells, and it was found that ^{125}I-radiolabelling did not appreciably affect the receptor affinity of the EMs [11]. We earlier prepared [^{3}H]Tyr^1-EM2 [12], and used it to determine directly the binding and signalling profile of EM2 in rat brain membranes [13]. Moreover, we and others have shown that EM1 and EM2 are capable of activating G-proteins and inhibiting adenylyl cyclase, and that all these effects are mediated by the μ-opioid receptors [14, 15].

The present paper describes the synthesis of specifically labelled radioisotopomers of EM1 and EM2, and demonstrates that the [^{3}H]EM2 radioisotopomers are not significantly different in receptor-binding studies, providing an excellent tool for further studies.

RESULTS AND DISCUSSION

We report here the preparation of EM1 labelled specifically on Tyr^1 and Pro^2, and EM2 labelled specifically on the Pro^2 and Phe^3 residues with high specific radioactivity. The radioligands were prepared by tritiation of the appropriate precursor peptides containing 3', 5'-diiodotyrosine (Dit) or 4'-iodophenylalanine or 3,4-dehydroproline (ΔPro). All the precursors could be separated from the tritiated products by both TLC and HPLC (*Tables 1* and *2*). The resolution of the ΔPro-containing precursors and the saturated EMs was low, and a long reaction time (120 min) was therefore employed to minimize the amount of the precursor in the

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crude product and to decrease the loss of the tritiated products during the purification step. However, the longer reaction time resulted in slight exchange reactions and therefore higher specific activity with increased specificity (*Table 3*). Thus, the Tyr and Phe residues also contained some tritium, but the Trp did not. This is the reason why the [^{3}H]Pro^2-EM1 had a specific activity higher than the theoretical value. In other cases, the specific activity attained was higher than 70 per cent of the theoretical value, and the specificity of the label was practically complete, resulting in satisfactory radioligands for metabolic studies and radioligand-binding experiments.

Before radioligand-binding studies, it is advisable to determine the biological half-life of a ligand within the used biological matrix. This is a quantitative property of a ligand which confirms its stability under the incubation conditions of the binding experiment. EM1 and EM2 exhibit long half-lives in the presence of rat brain membrane preparation (0.3 mg/cm^3 protein): 295 min and 230 min, respectively - therefore they do not degrade during the binding assays (see materials and methods).

Specific binding was found to be saturable with all five radioligands in saturation binding experiments. The equilibrium dissociation constant (K_d) and the maximal number of binding sites (B_{max}) were determined in order to characterize the differently radiolabelled EMs (*Table 4*). Linear regression analysis of the data after Scatchard transformation confirmed the existence of a single binding site (data not shown). The similarity of the biochemical data showed that the EM2 radioisotopomers were not significantly different in the radioreceptor assay, therefore the position of the labelling does not influence significantly the potency of the ligand. Preliminary data suggest that the different radioisotopomers of EM1 should behave in the same way as that of EM2 (data not shown).

EXPERIMENTAL

Materials

Protected and unprotected amino acids and resin were purchased from Calbiochem-Novabiochem or Bachem. The reagents used in the biological assays were provided by Sigma and Hoffmann-La Roche. Other reagents were from Merck or from Fluka. All

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reagents and solvents were of analytical or reagent grade and were used without further purification. The mobile phases of reversed-phase high-performance liquid chromatography (HPLC) for linear gradient elutions were mixed from 0.1% (v/v) trifluoroacetic acid (TFA) in water and 0.08% (v/v) TFA in acetonitrile. A Finnigan TSQ 7000 mass spectrometer was used to identify the peptide molecule ions under ESI ionization. Radio-TLC was performed on silica gel 60 F_{254}-precoated glass plates (Merck), and the radioactive spots were detected with a Berthold LB 2821 flow-through (9.9% methane/Ar) Geiger-Müller counter. Radio-HPLC was performed on a Jasco instrument, using a Vydac 218TP54 C_{18} (25×0.46 cm, 5 μm) column and liquid scintillation detection on a Canberra Packard Radiomatic 505TR Flow Radiochromatography Detector with the Ultima-Flo M scintillation cocktail. Tritiation was carried out in a self-designed vacuum manifold [16]. $^3H_2$ gas was purchased from Technobexport, Russia, and contained at least 98% tritium. The radioactivity of the tritiated compounds was measured with a Searle Delta 300 liquid scintillation counter in a toluene-Triton X-100 cocktail.

Methods

Synthesis of peptides on solid phase

Peptides were synthesized manually by using the Merrifield solid-phase method on 4-methylbenzhydrylamine resin. N^{α}-t-Boc chemistry with N-hydroxybenzotriazole (HOBt) and N,N'-dicyclohexylcarbodiimide (DCC) as coupling agents were employed for peptide elongation. The peptides were cleaved from the resin with anhydrous HF (10 cm^3/g resin) in the presence of anisole (1 cm^3/g resin) and dimethyl sulfide (1 cm^3/g resin) at 0 °C for 60 min. The peptide-resin mixtures were washed with diethyl ether to remove the scavengers, the peptides were then extracted with 30% acetic acid and the filtrate was lyophilized. The crude peptides were purified by HPLC on a Vydac 218TP1010 C_{18} (25×1 cm, 12 μm) column, using a linear gradient from 20% to 35% of the organic modifier within 25 min at a flow rate of 4 cm^3/min, with UV detection at 275 nm. Peptide purity was assessed by TLC and HPLC and the molecular weights of the peptides were established by ESI-MS (*Table 1*).

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Table 1. *Analytical data on the EM analogues*

Peptides	TLC*			HPLC#	MS◆	
	R_f(A)	R_f(B)	R_f(C)	k'	$[M+H]^+$	M_r
Tyr-Pro-Trp-Phe-NH_2	0.45	0.56	0.34	3.33	611.6	610
Dit-Pro-Trp-Phe-NH_2	0.63	0.46	0.42	4.83	863.0	862
Tyr-ΔPro-Trp-Phe-NH_2	0.52	0.50	0.37	2.70	609.3	608
Tyr-Pro-Phe-Phe-NH_2	0.43	0.55	0.37	2.83	572.7	571
Dit-Pro-Phe-Phe-NH_2	0.60	0.46	0.45	4.59	824.1	823
Tyr-ΔPro-Phe-Phe-NH_2	0.51	0.50	0.38	2.17	570.2	569
Tyr-Pro-Phe(pI)-Phe-NH_2	0.50	0.36	0.37	4.30	698.0	697

* R_f values on silica gel 60 F_{254}-precoated glass plates (Merck). Solvent systems: (A) acetonitrile/methanol/water (4:1:1), (B) 1-butanol/acetic acid/water (4:1:1), (C) ethyl acetate/pyridine/acetic acid/water (60:20:6:11). # Capacity factor for a Vydac 218TP54 C_{18} reversed-phase column (25×0.46 cm) with a gradient of 20% to 40% of organic modifier in 20 min. Flow rate 1 cm^3/min. All peptides were monitored at 216 nm. ◆ Measured and calculated molecular weights.

Tritium labelling

2 mg of precursor peptide dissolved in 1 cm^3 of dimethylformamide was tritiated in the presence of 1.5 μL of triethylamine (in the case of iodine-containing precursors) and 10 mg of PdO/$BaSO_4$ catalyst with tritium gas. Tritium gas was liberated from uranium tritide by heating, and 555 GBq (15 Ci) of it was introduced into the reaction vessel. The reaction mixture was stirred at room temperature for 80 min and the unreacted tritium gas was then adsorbed onto pyrophoric uranium. The catalyst was filtered off on a Whatman GF/C filter and the labile tritium was removed by repeated vacuum evaporation of an aqueous ethanolic solution of the radiolabelled product. The crude product was purified by HPLC to give a radioactive purity of >95%, checked by both TLC and HPLC (*Table 2*). The quantitative analysis of the pure, labelled peptides was performed by HPLC via a UV detector, using a calibration curve prepared with unlabelled EMs, and the total activity of the product was measured by liquid

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scintillation counting. The calculated specific activities (a) are given in *Table 2*. The tritiated peptides were dissolved in ethanol and were stored at a concentration of 37 MBq/cm^3 under liquid nitrogen.

Tritium distribution in labelled peptides

0.74 MBq of tritiated EM and 0.06 mg of unlabelled EM were hydrolysed for 24 h in 1 cm^3 of 6 M HCl at 110 °C under argon pressure in a closed ampoule. The solvent was removed by evaporation, and the samples were then dissolved in 1 cm^3 of 0.2 M borate buffer (pH 7.7). To 0.2 cm^3 of aqueous sample was added 0.2 cm^3 of 9-fluorenylmethyl chloroformate in acetone (15 mM). After about 45 sec, the mixture was extracted with pentane, and the aqueous phase was analysed by HPLC (*Table 3*).

Receptor-binding properties of [^{3}H]EM2 radioisotopomers

Saturation binding experiments were carried out according to the procedures published earlier [13] with slight modifications. The experiments were performed in glass tubes in 50 mM Tris.HCl buffer (pH 7.4) at 25 °C for 45 min in a final volume of 1 cm^3 in the presence of varying concentrations (between 0.01 nM and 20 nM) of [^{3}H]EM2 radioisotopomers. Nonspecific binding was determined by subtracting the values

Table 2. *Radioanalytical data on tritiated EMs*

Labelled peptides	a	TLC*			HPLC#
	TBq/mmol	R_f(A)	R_f(B)	R_f(C)	k'
[3',5'-$^3H_2$]Tyr-Pro-Trp-Phe-NH_2	1.53	0.45	0.56	0.33	3.32
Tyr-[3,4-$^3H_2$]Pro-Trp-Phe-NH_2	2.35	0.45	0.55	0.34	3.33
[3',5'-$^3H_2$]Tyr-Pro-Phe-Phe-NH_2	1.97	0.42	0.55	0.37	2.83
Tyr-[3,4-$^3H_2$]Pro-Phe-Phe-NH_2	1.88	0.42	0.55	0.37	2.84
Tyr-Pro-[4'-^{3}H]Phe-Phe-NH_2	0.77	0.42	0.56	0.38	2.83

*,# For conditions, see *Table 1*.

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obtained in the presence of 10^{-5} M naloxone. The displacement curves were analyzed with the software GraphPad Prism [17], using a nonlinear least squares algorithm. All experiments were carried out in duplicate assays and repeated at least four times.

Table 3. *Tritium distribution in radioligands*

Tritiated peptide	a/a_{max}*	HPLC#		
		Fmoc-[^{3}H]Tyr	Fmoc-[^{3}H]Pro	Fmoc-[^{3}H]Phe
H-[^{3}H]Tyr-Pro-Trp-Phe-NH_2	71%	99 %	-	-
H-Tyr-[^{3}H]Pro-Trp-Phe-NH_2	109%	7 %	83 %	10 %
H-[^{3}H]Tyr-Pro-Phe-Phe-NH_2	91%	99 %	-	-
H-Tyr-[^{3}H]Pro-Phe-Phe-NH_2	87%	1 %	91 %	8 %
H-Tyr-Pro-[^{3}H]Phe-Phe-NH_2	71%	2 %	-	98 %

Trp decomposes during strong acidic hydrolysis. * a/a_{max} is the ratio of the specific to the theoretically maximum specific activity. # Analysis on a Vydac 218TP54 C_{18} column (25x0.46 cm) with a gradient of 30% to 80% of organic modifier in 20 min. Flow rate 1 cm^3/min, λ= 216 nm.

Table 4. *Saturation binding studies of the radioisotopomers of EM2*

Radioligands	K_d (nM)	B_{max} (fmol/mg protein)
[3',5'-$^3H_2$-Tyr^1]EM2	0.68 ± 0.10	170.72 ± 24.87
[3,4-$^3H_2$-Pro^2]EM2	1.38 ± 0.22	174.67 ± 28.51
[4'-^{3}H-Phe^3]EM2	2.52 ± 0.66	121.39 ± 24.14

The values are the means ± S.E.M.

CONCLUSIONS

The specificity of the labelling is an extremely important aspect if metabolic studies are planned on the basis of the qualitative identification of the radioactive metabolites in the digestion mixture. Accordingly, we prepared specifically tritium-labelled EM1 and EM2 radioisotopomers with high specific radioactivity. The specificity of the labelling was satisfactory for detailed examinations of the metabolism of the

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endomorphins, and the specific activities were sufficient for radioligand-binding assays. The EM2 radioisotopomers proved equipotent in saturation binding experiments. Thus, the position of the tritium label does not influence the ligand-receptor interaction, and there is no biochemical isotopic effect between EM2 and the μ-opioid receptors.

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II

Cs. Tömböly, M. Spetea, A. Borsodi and G. Tóth

"Synthesis of Tritium Labelled Endomorphin-2 and its Stability in the Radioreceptor Assay."

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SYNTHESIS OF TRITIUM LABELLED ENDOMORPHIN II AND ITS STABILITY IN THE RADIORECEPTOR ASSAY

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Endomorphine I (Tyr-Pro-Trp-Phe-NH_2) and endomorphin II (Tyr-Pro-Phe-Phe-NH_2) are recently isolated neuropeptides. They have the highest specificity and affinity for the μ-opiate receptor among all the endogenous substances so far described, and they may be natural ligands for this receptor [1]. We prepared the [^{3}H]endomorphin II with high specific radioactivity (53.4 Ci/mmol) by catalytic dehalotritiation. The precursor [(3,5-I_2)Tyr^1]-endomorphin II was synthesized by solid phase peptide synthesis using Boc chemistry. Labelled endomorphin II was used to investigate its binding properties in rat brain membrane. The stability of [^{3}H]endomorphin II toward enzymatic degradation in membrane preparation was examined by RP-HPLC and by using a radioactivity detector.

1 Introduction

In 1806, Sertürner isolated the main pharmacologically active alkaloid of the opium and named it morphine after the god of the dreams, Morpheus [2]. Morphine became the most powerful analgesic for the treatment of several pains. Since the 1970s, the attempts to determine its mechanism of action led to the identification of opioid receptors and the isolation and characterization of endogenous opioid peptides. The results of pharmacological and biochemical studies suggested the existence of three types of receptors named μ-, δ- and κ-opiate receptor. These types of receptors differ in their ligand selectivity and anatomical distribution. Endomorphin I and endomorphin II have been isolated recently from bovine brain [1] and in a relatively large amount from human brain cortex [3]. Endomorphin II was synthesized earlier too [4] as a morphiceptin analogue with high affinity and selectivity for μ-opioid receptors. These new peptides have the highest specificity and affinity for the μ-opiate receptor among all the endogenous substances so far described and they may be natural ligands for this receptor. We prepared [^{3}H]endomorphin II with high specific radioactivity (53.4 Ci/mmol) by the catalytic dehalotritiation of [(3,5-I_2)Tyr^1]-endomorphin II and it was used to investigate its binding properties in rat brain membrane. Furthermore, the stability of [^{3}H]endomorphin II toward enzymatic degradation in membrane preparation was examined.

2 Experiment

Materials

All reagents and solvents were analytical or reagent grade and were used without further purification. Protected and unprotected amino acids and resin were purchased from Aldrich Chemical Co. or Bachem Feinchemicalien or from Novabiochem. Coupling agents were from Richelieu Biotechnologies, or from Fluka. Trifluoroacetic acid (TFA) was from Fisher Scientific.

Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} precoated glass plates. The plates and solvents were from Merck. The following solvent systems were used for TLC analysis: (I): butanol-acetic acid-water (4:1:1) and (II): acetonitrile-methanol-water (4:1:1). UV light, iodine vapour and ninhydrin (1%, Merck) were employed to detect the peptides.

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Merck-Hitachi RP-HPLC system utilizing a Vydac 218TP1010 C-18 (25 × 1 cm, 12 μm) semipreparative column for preparative purposes, and a Vydac 218TP54 C-18 (25 × 0.46 cm, 5 μm) analytical column for analytical purposes.

The mass of the molecule ions was determined by mass spectrometry (Finnigan TSQ 7000) by ESI ionisation. Tritiation reaction was carried out on a self-designed vacuum manifold [5]. $^3H_2$gas was purchased from Technobexport, Russia and contained at least 98% tritium. $PdO/BaSO_4$ (10% Pd) (Merck) was used as a catalyst of dehalotritiation.

The radiochemical purity of the labelled peptide was checked by TLC, detected with a Berthold Radiochromatogram Tracemaster. Radioactivity was counted in a toluene-Triton X- 100 scintillation cocktail with a SearleDelta300 liquid scintillation counter (LSC).

The analysis and the degradation of tritium-labelled material was performed on a RP-HPLC (Jasco) instrument, using a Merck 50943 LiChroCART (125-4 LiChrospher 100 RP-18,5 μm) column, and UV-detection on a Jasco UV-975 spectrometer and liquid scintillation detection on a Canberra Packard 505 TR Flow Radiochromatography Detector. Reagents of the biological assays were provided by Sigma Chemicals and Hoffmann-La Roche.

Synthesis of [(3,5-I_2)Tyr^1]-endomorphin II

The synthesis of the precursor compound was performed manually by solid phase peptide synthesis on 4-methyl-benzhydrylamine resin. N^{α}-t-butyloxycarbonyl (Boc) protected amino acids were used throughout. N,N'-Dicyclohexylcarbodiimide and N-hydroxybenzotriazole were used in the active ester method. All the coupling reactions were monitored by ninhydrin test. The Boc protecting groups were removed with trifluoroacetic acid (TFA). The peptide was cleaved from the resin with anhydrous HF (10 mL/g resin) with anisole added as a scavenger (1 mL/g resin) for 60 min at 0 °C. The peptide was extracted from the resin by first washing with ethylether and then stirring of the resin under glacial acetic acid. The resin was filtered off, washed with 30 % acetic acid and the filtrate was lyophilized. The crude peptide was purified by RP-HPLC using linear gradient starting with 20% CH_3CN in 0.1% aqueous TFA 0.6%/min for 25 minutes at a flow rate of 4 mL/min with UV detection at 275 nm. The purity was determined by TLC in two solvent systems (I) and (II) on silica gel 60 and by analytical RP-HPLC: R_f(I) = 0.59, R_f(II) = 0.62, k' = 3.5. The $[M+H]^+$ molecular ion and fragmentation patterns were obtained by ESI-MS and it was in agreement with the calculated molecular weight of the peptide: $[M+H]^+$ = 824.1 (calculated 824).

Synthesis of [H] endomorphin II

2 mg of [(3,5-I_2)Tyr^1]-endomorphin II was dissolved in 1 mL dimethylformamide and 1.5 μL of triethylamine and 10 mg of $PdO/BaSO_4$ catalyst was added. After putting a magnetic stirrer into the reaction vessel, it was connected to the tritiation apparatus. The solution was frozen in liquid nitrogen, and the apparatus was evacuated. Tritium gas was liberated from uranium tritide by heating, and 555 GBq (15 Ci) of that was

introduced into the reaction vessel. The frozen solvent was warmed and stirred at room temperature for 80 min. The reaction mixture was frozen again and the unreacted tritium was adsorbed onto pyrophoric uranium. The catalyst was filtered on a Whatman GF/C filter and then washed with ethanol. The labile tritium was removed by repeated vacuum evaporation of water: ethanol (1:1) solution from the radiolabelled product. The total activity of the crude material was measured by LSC and was determined to be 3.46 GBq (93.5 mCi). The purification of the crude product was made by preparative TLC using the solvent system (I) on silica gel 60. The peptide was washed out from the silica by ethanol. The purity was checked by TLC: $R_f(I) = 0.65$, $R_f(II) = 0.46$, and analytical RP-HPLC using linear gradient starting with 20% CH_3CN in aqueous TFA 0.6%/min for 25 min at flow rate of 1 mL/min: k' = 6.94. Quantitative analysis of the amount and radioactivity of labelled peptide was performed by RP-HPLC via UV and radioactivity detector by using calibration curve made by unlabelled endomorphin II and the specific activity of tritiated endomorphin II was 1.98 TBq/mmol (53.4 Ci/mmol). The labelled peptide was dissolved in ethanol and was stored in 2 mL aliquots (37 MBq/mL) under liquid nitrogen, and its purity was checked before using in biological assays and repurified if necessary.

Synthesis of endomorphin I and II

Endomorphin I and II were prepared by a solid phase techinque utilizing Boc chemistry, as above in the case of [(3,5-I_2)Tyr^1]-endomorphin II. These peptides were used in the radioreceptor assay.

Membrane preparations

A crude membrane fraction was isolated from Wistar rat brains according to the method previously described [6]. Rat brains were homogenized on ice in 50 mM Tris. HCl buffer (pH 7.4) then centrifuged at 40000 g for 20 min at 4°C. The resulting pellets were resuspended in fresh Tris. HCl buffer, incubated at 37°C for 30 min and recentrifuged. The final pellets were suspended in 50 mM Tris. HCl (pH 7.4) containing 0.32 M sucrose and were stored under liquid nitrogen until use. Protein concentration was determined by the method of Bradford [7].

Stability of the radioligand in rat brain membrane preparation

The stability of [^{3}H]endomorphin II toward enzymatic degradation in rat brain membrane preparation was examined by incubating the radioligand (5 μCi) with a membrane suspension (400 μg protein) for 45 min at 25 °C in a final volume of 1 mL in the presence and absence of peptidase blockers. At the end of the incubation, the membrane suspension was centrifuged and the aliquot of supernatant (200 μL) was analyzed by RP-HPLC via UV and radioactivity detector using a linear gradient of 10-35% CH_3CN in 0.1% TFA in water within 25 min at a flow rate of 1 mL/min. All receptor binding experiments were performed in an assay buffer consisting of 50 mM Tris. HCl, 1 mg/mL bovine serum albumin, 50 μg/mL bacitracin, 10 μM captopril and 0.1 mM phenylmethanesulfonyl-fluoride (PMSF).

3 Results and discussion

We reported the preparation of [3H]-endomorphin II with 1.98 TBq/mmol (53.4 Ci/mmol) of specific activity. The specific acticity of [^{3}H]endomorphin means that

approximately two tritium atoms are incorporated per molecule. The chromatographic run showed 96.6% purity of the tritiated endomorphin II in the absence of membrane fraction, while in the presence of membrane about 60% intact radioligand was recovered. When peptidase blockers were present during incubation with rat brain membranes no significant degradation had occurred. Association rate constant (k_{+1}), dissociation rate constant (k–1), equilibrium dissociation constant (K_d) and maximal number of binding sites (B_{max}) values were determined by kinetic and binding studies and found to be 0.088 ± 0.008 $min^{-1}nM^{-1}$, 0.085 ± 0.001 min^{-1}, 1.12 ± 0.25 nM, 114.80 ± 19.57 fmol/mg protein, respectively. The detailed description of binding assay will be published elsewhere. These data and the competition binding studies suggest that [^{3}H]-èndomorphin II should be a very promising tool for identification and characterization of the μ-opioid receptors in various tissues.

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III

A. Péter, G. Tóth, **Cs. Tömböly**, G. Laus and D. Torwé

"Liquid Chromatographic Study of the Enzymatic Degradation of Endomorphins, with Identification by Electrospray Ionization Mass Spectrometry."

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Liquid chromatographic study of the enzymatic degradation of endomorphins, with identification by electrospray ionization mass spectrometry

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Abstract

The recently discovered native endomorphins play an important role in opioid analgesia, but their metabolic fate in the organism remains relatively little known. This paper describes the application of high-performance liquid chromatography combined with electrospray ionization mass spectrometry to identify the degradation products resulting from the incubation of endomorphins with proteolytic enzymes. The native endomorphin-1, H–Tyr–Pro–Trp–Phe–NH_2 (1), and endomorphin-2, H–Tyr–Pro–Phe–Phe–NH_2 (2), and an analog of endomorphin-2, H–Tyr–Pro–Phe–Phe–OH (3), were synthetized, and the levels of their resistance against carboxypeptidase A, carboxypeptidase Y, aminopeptidase M and proteinase A were determined. The patterns of peptide metabolites identified by this method indicated that carboxypeptidase Y first hydrolyzes the C-terminal amide group to a carboxy group, and then splits the peptides at the Trp^3–Phe^4 or Phe^3–Phe^4 bond. The remaining fragment peptides are stable against the enzymes investigated. Carboxypeptidase A degrades only analog 3 at the Phe^3–Phe^4 bond. Aminopeptidase M cleaves the peptides at the Pro^2–Trp^3 or Pro^2–Phe^3 bond. The C-terminal fragments hydrolyze further, giving amino acids and Phe–NH_2-s while the N-terminal part displays a resistance to further aminopeptidase M digestion. Proteinase A exhibits a similar effect to carboxypeptidase Y: the C-terminal amide group is first converted to a carboxy group, and one amino acid is then split off from the C-terminal side. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Electrospray ionization; Endomorphins; Enzymes; Peptides

1. Introduction

Endomorphin-1 (1; H–Tyr–Pro–Trp–Phe–NH_2) and endomorphin-2 (2; H–Tyr–Pro–Phe–Phe–NH_2) have recently been isolated from bovine brain [1] and human brain [2] (Fig. 1). Both peptides are highly potent and selective μ-opioid receptor ago-

H-Tyr-Pro-Trp-Phe-NH_2 (1)

H-Tyr-Pro-Phe-Phe-NH_2 (2)

H-Tyr-Pro-Phe-Phe-OH (3)

Fig. 1. Endomorphin analogs studied: endomorphin-1 (1); endomorphin-2 (2); and an analog of endomorphin-2 (3).

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nists. Peptide 1 exerts potent bioactivity both in vitro and in vivo, producing prolonged analgesia in mice after central administration. Stone et al. [3] observed short-lasting antinociceptive effects of 1 and 2. The short duration of action suggests that these peptides are unstable in the spinal cord. Spetea et al. [4] described the binding characteristics of [^{3}H]endomorphin-2 in a rat brain membrane preparation and used peptidase inhibitors in a radioligand binding assay. In the absence of peptidase inhibitors 40% of the radioligand in the incubation mixture was destroyed after incubation for 45 min at 25°C. These different findings highlight the importance of studying the stabilities of 1 and 2 against enzymatic degradation.

The cerebrospinal fluid and central nervous system contain amino-, endo- and carboxypeptidases capable of processing and/or degrading opioid peptides, and altered rates of degradation have been reported in some pathologic conditions [5,6].

This paper describes the application of high-performance liquid chromatography (HPLC) combined with electrospray ionization mass spectrometry (ESI-MS) [6,7] to identify the degradation products of these endomorphins after incubation with neuropeptide-degrading proteases. Peptides 1 and 2 and a modified analog of 2 (3; H–Tyr–Pro–Phe–Phe–OH) were incubated with carboxypeptidase A, carboxypeptidase Y, aminopeptidase M and proteinase A.

Carboxypeptidase A, a metallopeptidase, removes C-terminal aromatic and long side-chain aliphatic residues most rapidly [8]. Native endomorphins, being amidated peptides, are probably stable against carboxypeptidase A digestion, but analog 3 could be a substrate.

Carboxypeptidase Y is a Ser-type enzyme, and accepts a broad spectrum of substances. Ser carboxypeptidase does not require a free C-terminal carboxylate group. It can catalyse the hydrolysis of peptide esters and amides [9]. Additionally, Ser carboxypeptidase acts on peptide amides, releasing either ammonia (amidase activity) or amino acid amides (dipeptidyl amino acid amide hydroxylase activity) [10]. Carboxypeptidase Y exhibits a preference for peptides containing hydrophobic amino acid residues (except Pro).

Aminopeptidases are present in the brain and peripheral tissues and destroy the activity of opioid peptides [11]. Aminopeptidase M releases an N-terminal amino acid from peptides, amides or arylamides. The amino acid released is preferably Ala, but most amino acids including Pro (slow reaction) may also be involved. When a terminal hydrophobic residue is followed by a Pro residue, the two may be released as an intact dipeptide [12]. Aminopeptidase cleaves opioid peptides into inactive fragments by splitting off Tyr [13]. As concerns dynorphins, two aminopeptidases that could participate in N-terminal amino acid removal were identified in the rat brain: the puromycin-sensitive aminopeptidase and aminopeptidase M [14].

Proteinase A is a nonpepsin-type acid endopeptidase; it cleaves small peptides at only one site and the rates of hydrolysis differ markedly among them. It also cleaves amidated peptides, e.g., Substance P and its analogs degrade rapidly [15].

2. Experimental

2.1. Chemicals and reagents

Peptides 1 and 2 were prepared by solid-phase synthesis on a 4-methylbenzhydrylamine resin by means of Boc chemistry, and 3 was prepared by using Merrifield resin, also with Boc protection (Fig. 1) (nomenclature and abbreviations are in accordance with the IUPAC-IUB Joint Commission of Biochemical Nomenclature (JCBN) recommendations [16]). The synthetic peptides were purified on a Merck–Hitachi HPLC system.

Proteinase A (endopeptidase, from baker's yeast; EC 3.4.23.6), carboxypeptidase A (type II DFP from bovine pancreases, treated with diisopropylfluorophosphate to eliminate trypsin and chymotrypsin activity; EC 3.4.17.1,), carboxypeptidase Y (from baker's yeast; EC 3.4.16.1) and aminopeptidase M (leucine aminopeptidase, type IV-S: from porcine kidney microsomes; EC 3.4.11.2) were obtained from Sigma–Aldrich (Steinheim, Germany). The homogenity of each of these commercially available enzymes was checked by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Different batches of enzymes were checked, and only enzymes giving only one protein band were used in digestion measurements. Tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl), tri-

fluoroacetic acid (TFA) of analytical-reagent grade, acetonitrile and methanol of HPLC grade and other reagents of analytical-reagent grade were purchased from Merck (Darmstadt, Germany). Buffers were prepared with Milli-Q water and were further purified by filtration on a 0.45-μm filter type HV (Millipore, Molsheim, France).

2.2. Apparatus

Analytical and semipreparative chromatography were performed on a chromatographic system consisting of an L-6200A Intelligent pump, an L-3000 multichannel photo detector, a T-6300 column thermostat, and a D-6000 HPLC Manager with interface (Merck–Hitachi, Darmstadt, Germany). The samples were introduced via a Rheodyne Model 7125 valve (Cotati, CA, USA) equipped with a 20-μl loop.

The column used was a Vydac 218TP54 C_{18} (250×4.6 mm I.D.), 5-μm particle size (Separations Group, Hesperia, CA, USA). Gradient elutions were run with a 0.1% aqueous solution of TFA as mobile phase A and 0.1% TFA in acetonitrile as phase B, ranging from 0% to 50% B within 20 min.

2.3. Enzymatic cleavage assay

2.3.1. Preparation of solutions

Solutions of peptides 1–3 were made by dissolving them in 50 m*M* Tris·HCl (pH 7.4) buffer to give a final concentration of 1 m*M*.

Carboxypeptidase A and aminopeptidase M were used in their original concentrations, 18 and 10 mg protein/ml, respectively. Carboxypeptidase Y solution (0.5 mg/ml) was made by dissolving 0.16 mg enzyme in 320 μl 50 m*M* Tris·HCl buffer (pH 7.4). Proteinase A solution (0.5 mg/ml) was made by dissolving 0.2 mg enzyme in 400 μl 50 m*M* Tris·HCl buffer (pH 7.4) containing 10% (v/v) glycerol.

2.3.2. Enzymatic digestion

To 1000 μl 50 m*M* Tris·HCl buffer, 200 μl peptide solution was added, and the reaction mixture was incubated for 30 min at 37°C in a Julabo SW-200 agitating water bath (Julabo Labortechnik, Seelbach, Germany). After preincubation, one of the enzymes was added and the mixture was kept at 37°C. (The volumes of added enzyme solutions were as follows: 10 μl aminopeptidase M, 10 μl carboxypeptidase A, 30 μl carboxypeptidase Y or 30 μl proteinase A.) At designated intervals, 20 μl aliquots were removed and quenched in 20 μl 0.1 *M* HCl. The time interval of sampling was chosen so that a kinetic curve could be constructed. Samples were analyzed by HPLC for the quantitative determination of digestion products. For LC–ESI-MS analysis, the reaction was stopped at a degree of conversion of about 50%, and the whole reaction mixture was used for prepurification on Sep-Pak cartridges.

2.3.3. Sample handling and data processing

For ESI-MS and HPLC–ESI-MS analysis the digested samples were prepurified on C_{18} cartridges. For this purpose, the digested sample was taken up in an 0.1% aqueous solution of TFA and adsorbed on prewetted Sep-Pak C_{18} cartridges (Millipore, Bedford, USA) [17]. The cartridge was washed with 0.1% TFA and peptides were eluted with 0.1% TFA–acetonitrile (20:80, v/v).

Blanks were run by incubating the enzyme and the enzyme-free reaction mixture for several hours at 37°C, and the products formed were taken into account.

The results were calculated by taking into account the amount of starting peptide consumed and the amounts of degradation fractions produced, and are given in area% calculated from the HPLC measurements.

2.4. Mass spectrometry

The MS measurements were carried out on a Quattro II apparatus (Micromass UK, Altrincham, UK) with ESI and coupled to an HPLC system. The HPLC system consisted of a low-pressure gradient pump type 325, a UV detector type 332 and an Autosampler Type 465, all from Kontron (Milan, Italy). The column used was a Vydac 218TP54 C_{18} (250×4.6 mm I.D.), 5-μm particle size. The effluent of the HPLC column was split by an LC Packings split system with a split ratio of 1 to 10. For loop injection analysis, a Harvard Model 22 syringe infusion pump (South Natick, MA, USA) and a Rheodyne 7125 injector with a 50-μl loop were used. The flow-rate of the syringe pump was kept at 30 μl/min.

The mass spectrometer was operated under the

following conditions: capillary voltage 3.5 kV; cone voltage 50 V; scan duration 0.5 s (data type: compressed centroid); source temperature 60°C; mass range 110 to 1100. Data were acquired in positive-ion mode and the spectra were recorded with a Mass Lynx Application Software System (Micromass UK, Wythenshawe, UK). Peptides were identified via their molecular ions. Identification of the fragments by the direct application of MS to the digestion mixture was not possible. The on-line LC–ESI-MS was applied for identification of the peptide fragments. An example of the identification of the fragments of 1 after digestion with carboxypeptidase Y and HPLC fractionation is shown in Fig. 2.

3. Results and discussion

Four different enzymes were chosen to investigate their effects on the stabilities of endomorphins. Carboxypeptidase Y can catalyze the hydrolysis of amidated peptides and may release ammonia in consequence of its amidase activity [9,10]. The accommodation of an amino acid amide is probably aided by a beneficial hydrogen-bond between the C-terminal NH_2 group of the substrate and the deprotonated form of Glu-145 of the enzyme [10]. Endomorphins 1 and 2 were good substrates of enzymatic digestion with carboxypeptidase Y.

The results of HPLC and MS analyses of enzymatic digestion with carboxypeptidases are presented in Table 1 and Figs. 3 and 4. Fig. 3 relates to the separation of the degradation fragments and the starting peptides. The sequence of elution of the fragments could be rationalized in terms of their hydrophobicities. Fragments containing Trp in position 3 always had higher retention times than the corresponding fragments containing Phe in position 3. As concerns the separation of fragments containing a C-terminal amide or hydroxy group, the former are more hydrophilic and have shorter retention times.

For 1 and 2, three major peaks appeared in the chromatogram after digestion with carboxypeptidase Y (Fig. 3). The fragments observed indicate that the digestion of 1 and 2 with carboxypeptidase Y took place in two steps. In the first step, the C-terminal amide group underwent hydrolysis to the carboxy group; then, in the second step, cleavage of the Trp^3–Phe^4 or Phe^3–Phe^4 bond occurred:

H-Tyr-Pro-Trp-Phe-NH_2	H-Tyr-Pro-Phe-Phe-NH_2	(1)
↓	↓	
H-Tyr-Pro-Trp-Phe-OH	H-Tyr-Pro-Phe-Phe-OH	(2)
↓	↓	
H-Tyr-Pro-Trp-OH + Phe	H-Tyr-Pro-Phe-OH + Phe	(3)

The total degradation of 1 requires more than 20 min, whereas 2 was no longer present after 5 min. The remaining fragment peptides (H–Tyr–Pro–Trp–OH or H–Tyr–Pro–Phe–OH) are stable: even after hydrolysis for 20 h, no sign of new products could be observed in the chromatogram. Results concerning the stabilities of the peptides against carboxypeptidase Y digestion are presented in Table 1. The half-lives ($t_{1/2}$) for 1 and 2 were calculated from the rate constants (k), as $0.693/k$, using the kinetic curves of consumption of the peptides, and were about 3.0 and 1.0 min, respectively.

Endomorphins 1 and 2, being amidated peptides, exhibited high stability against enzymatic digestion with carboxypeptidase A: no sign of degradation could be observed after digestion for 24 h at 37°C. Analog 3 degrades rapidly in the presence of carboxypeptidase A, with a half-life of less than 5 s, and gives two major products: H–Tyr–Pro–Phe–OH and Phe (Table 1). This behavior is in agreement with literature data; carboxypeptidase A truncates peptides from the C-terminal side, releasing one amino acid [8].

Aminopeptidases release an N-terminal amino acid from the peptide or peptide amide [11]. The present endomorphins were split between Pro^2–Trp^3 or Pro^2–Phe^3 (Table 2). This unusual finding can be explained in terms of the Pro content of the molecules. Neubert et al. [12] showed that β-casomorphins, which are Pro-rich peptides, are very resistant to proteases. For their degradation study, they used dipeptidyl peptidase IV, which belongs in the aminopeptidase family, and splits off dipeptides from the N-terminal end of the peptides. The literature data indicate that, when an N-terminal hydrophobic residue is followed by a Pro residue, the two amino acids may be released as an intact dipeptide by aminopeptidase M, as was observed in our case [18].

The total degradation of 1 requires more than 1 h,

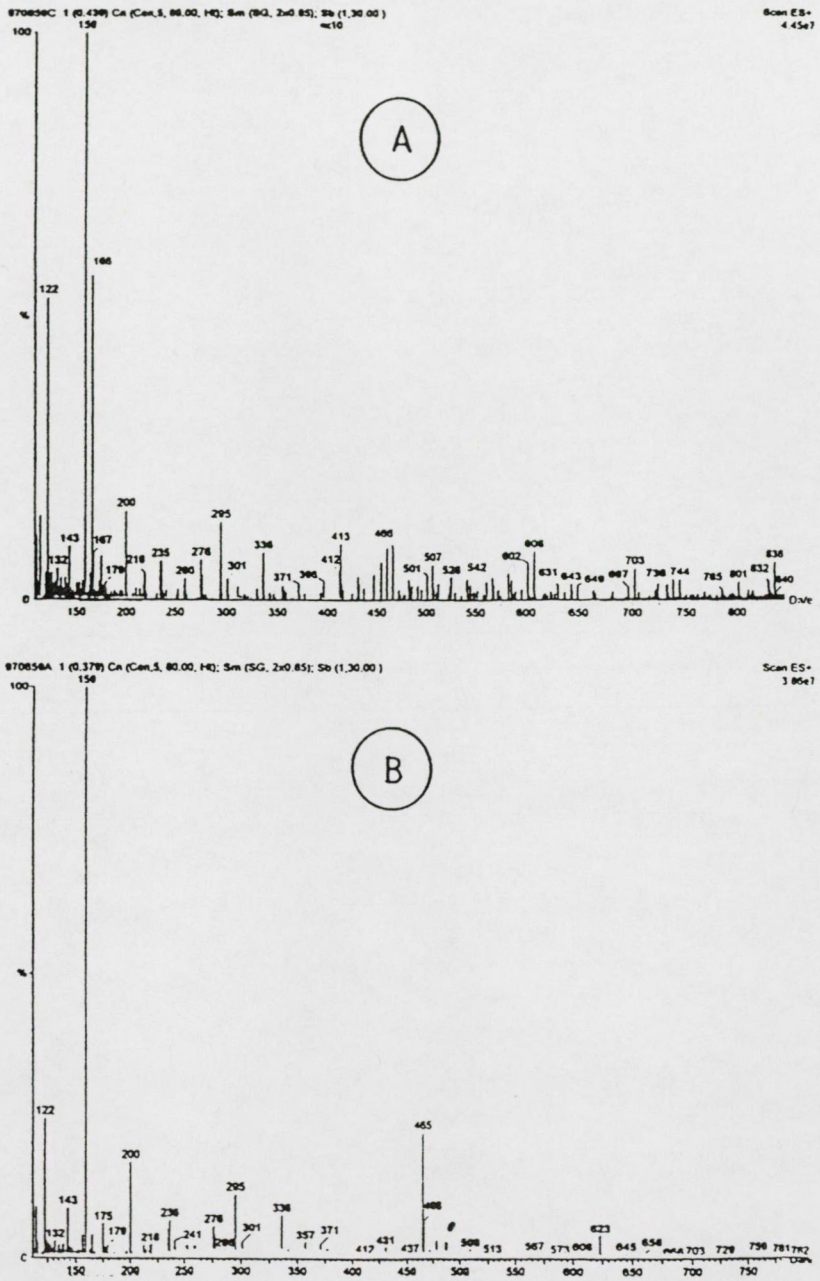

Fig. 2. Mass spectra of fragments of 1 after enzymatic digestion with carboxypeptidase Y and HPLC fractionation. Composition of digested mixture: 1, 166 μM; carboxypeptidase Y, 12.5 μg protein/ml. Incubation time, 15 min. (A) Phe, $m/z=166$; (B) H–Tyr–Pro–Trp–OH, $m/z=465$; (C) H–Tyr–Pro–Trp–Phe–OH, $m/z=612$.

while 2 had disappeared from the reaction mixture after a digestion period of 3 h. The peptide fragments formed displayed different stabilities. The resulting C-terminal dipeptide fragments, H–Trp–Phe–NH_2 or H–Phe–Phe–NH_2, were unstable and hydrolyzed further. They were split into the corresponding

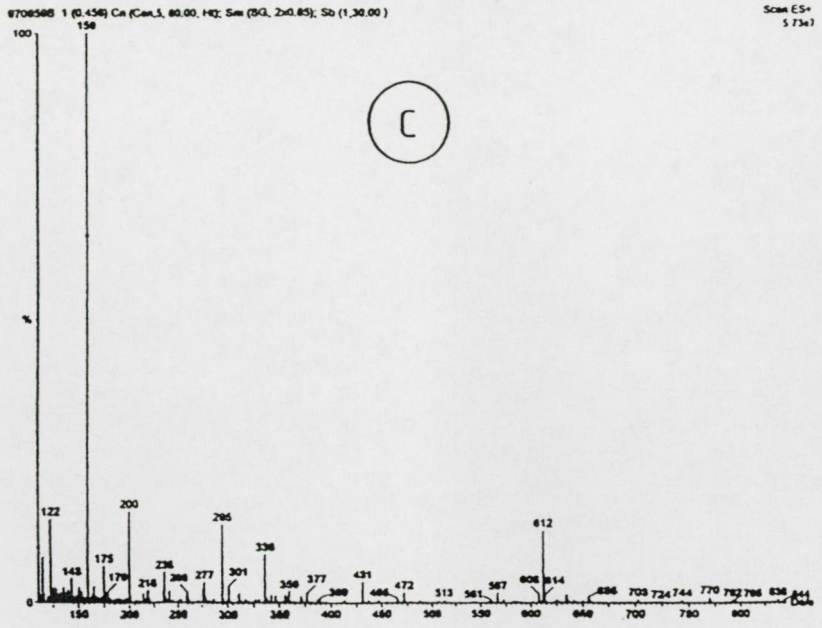

Fig. 2. (*continued*)

Table 1
Main degradation products of 1 and 2 on digestion with carboxypeptidase Y, and of analog 3 with carboxypeptidase A

Endomorphin	t_r[a]	m/z[b]	$M_{r(calc)}$[c]	Sequence assignment	Sequence no.	$t_{1/2}$[d] (min)
1	9.8	166	165.1	Phe	4	
	15.2	465	464.3	H–Tyr–Pro–Trp–OH	1–3	
	17.9	612	611.4	H–Tyr–Pro–Trp–Phe–OH	1–4	
	17.1	611	610.4	H–Tyr–Pro–Trp–Phe–NH_2 (intact peptide)	1–4	3.1
2	9.8	166	165.1	Phe	4	
	14.9	426	425.3	H–Tyr–Pro–Phe–OH	1–3	
	17.6	573	572.4	H–Tyr–Pro–Phe–Phe–OH	1–4	
	16.6	572	571.4	H–Tyr–Pro–Phe–Phe–NH_2 (intact peptide)	1–4	1.0
3	9.8	166	165.1	Phe	4	
	14.9	426	425.3	H–Tyr–Pro–Phe–OH	1–3	
	17.6	573	572.4	H–Tyr–Pro–Phe–Phe–OH (intact peptide)	1–4	<0.1

[a] HPLC retention time in min. HPLC conditions: column, Vydac 218TP54 C_{18}; gradient elution, 50% B in 20 min, see Experimental; detection, 210 nm; flow-rate, 0.8 ml/min.

[b] m/z Values for the ion $[M+H]^+$ in the mass spectra.

[c] Monoisotopic masses.

[d] Half-lives of peptides in the presence of carboxypeptidase Y and carboxypeptidase A.

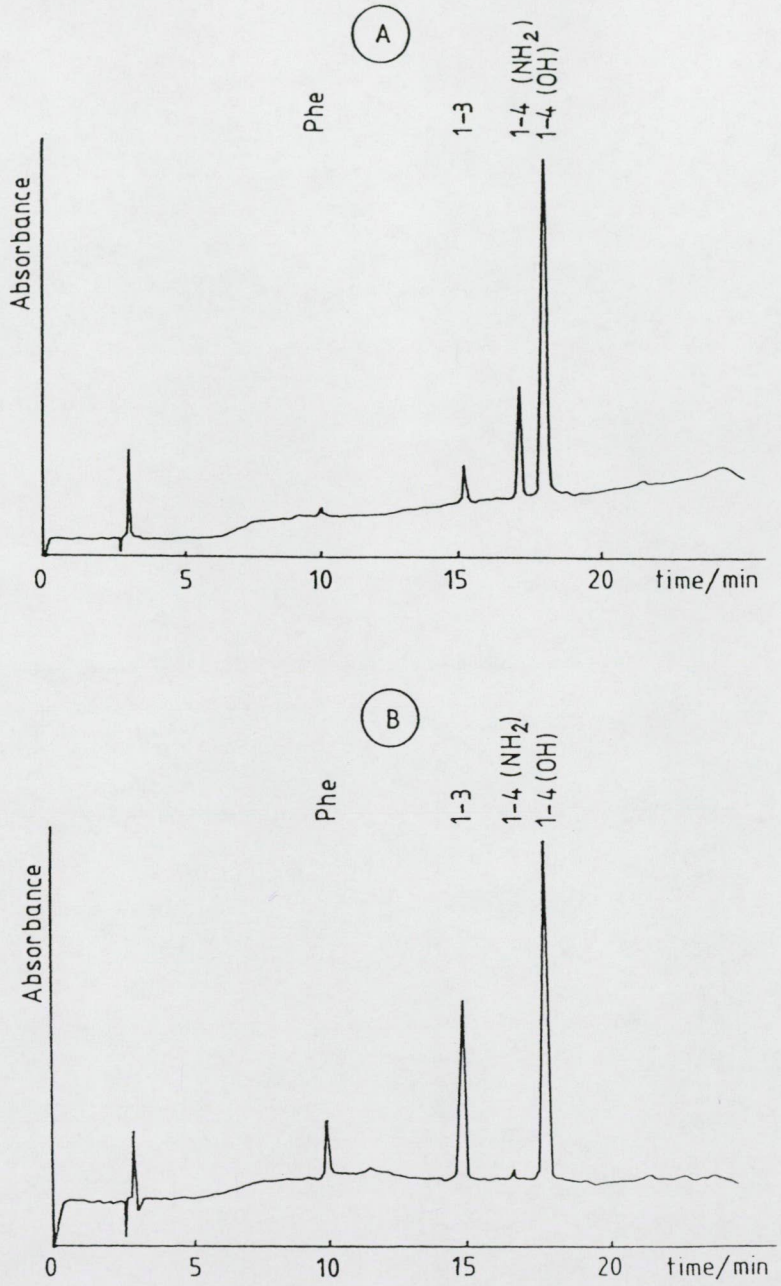

Fig. 3. HPLC separation of mixtures of products of degraded endomorphins after digestion with carboxypeptidase Y. (A) 1; (B) 2. Composition of digestion mixture: 1, 166 μM; 2, 166 μM; Carboxypeptidase Y, 12.5 μg/ml. Incubation time, 15 min; conditions of analysis: column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 210 nm; gradient elution, 50% B in 20 min, see Experimental. Peaks: see Table 1.

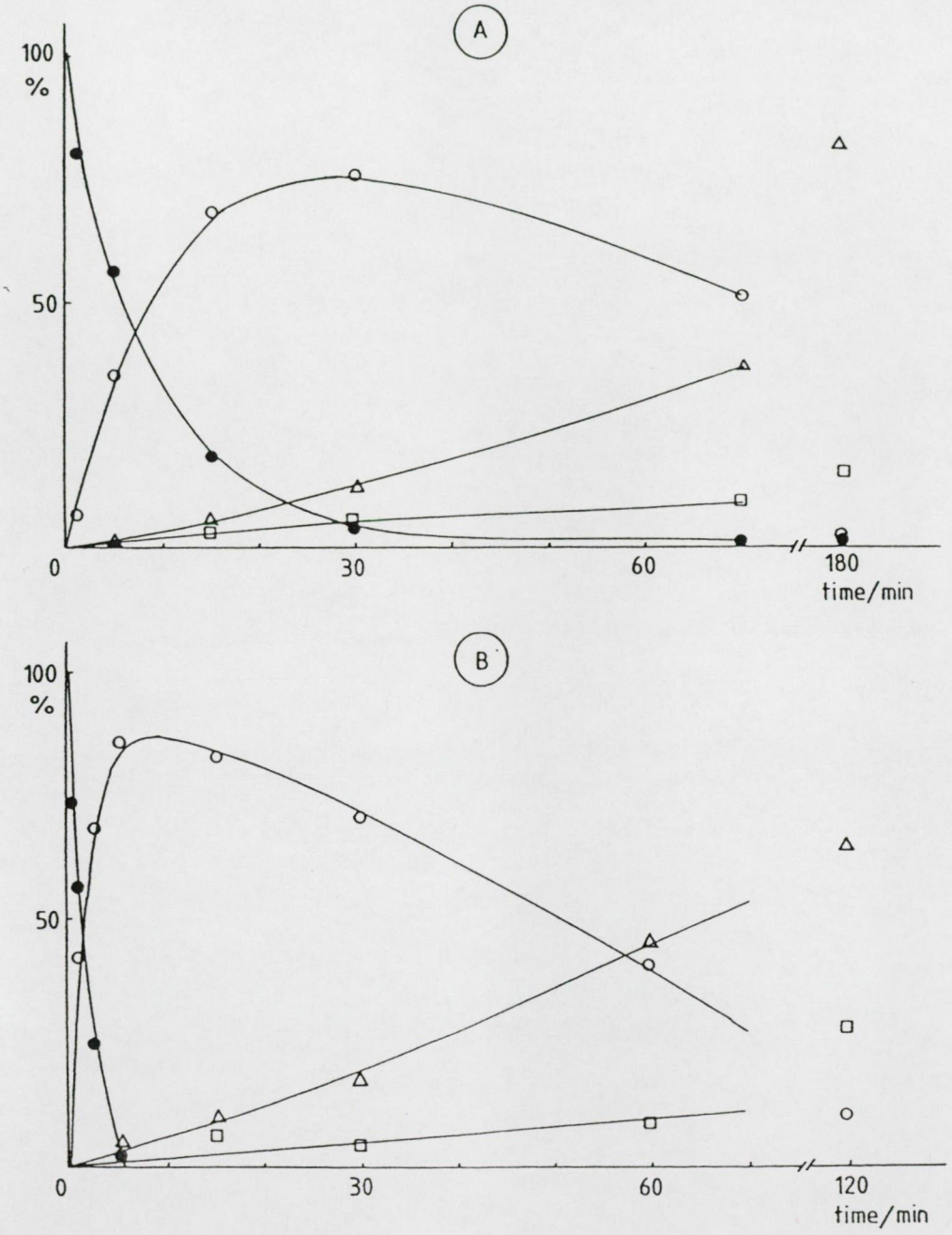

Fig. 4. Rates of change of peak intensities of the starting peptides and digestion products of endomorphins on incubation with carboxypeptidase Y. (A) 1; (B) 2. Composition of digestion mixture: 1, 166 μM; 2, 166 μM; Carboxypeptidase Y, 12.5 μg/ml; Conditions of analysis: column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 210 nm; gradient elution, 50% B in 20 min, see Experimental. ● (A,B) starting peptide; ○ (A) H–Tyr–Pro–Trp–Phe–OH; (B) H–Tyr–Pro–Phe–Phe–OH; △ (A) H–Tyr–Pro–Trp–OH; (B) H–Tyr–Pro–Phe–OH; □ (A,B) Phe.

Table 2
Main degradation products of 1 and 2 on digestion with aminopeptidase M

Endomorphin	t_r[a]	m/z[b]	$M_{r(calc)}$[c]	Sequence assignment	Sequence no.	$t_{1/2}$[d] (h)
1	9.0	165	164.1	Phe–NH_2	4	
	10.0	279	278.2	H–Tyr–Pro–OH	1–2	
	11.9	205	204.1	Trp	3	
	13.7	351	350.2	H–Trp–Phe–NH_2	3–4	
	17.1	611	610.4	H–Tyr–Pro–Trp–Phe–NH_2 (intact peptide)	1–4	0.17
2	9.0	165	164.1	Phe–NH_2	4	
	9.8	166	165.1	Phe	3	
	10.0	279	278.2	H–Tyr–Pro–OH	1–2	
	13.4	312	311.2	H–Phe–Phe–NH_2	3–4	
	16.6	572	571.4	H–Tyr–Pro–Phe–Phe–NH_2 (intact peptide)	1–4	0.25

[a] HPLC retention time in min. HPLC conditions: column, Vydac 218TP54 C_{18}; gradient elution, 50% B in 20 min, see Experimental; detection, 210 nm; flow-rate, 0.8 ml/min.
[b] m/z Values for the ion $[M+H]^+$ in the mass spectra.
[c] Monoisotopic masses.
[d] Half-lives of peptides in the presence of aminopeptidase M.

amino acids (Trp or Phe) and amino acid amide (Phe–NH_2) within 1 h of the start of digestion. The N-terminal part of the hydrolysis product (H–Tyr–Pro–OH) was relatively stable; Tyr appeared in the chromatogram after 5 h. The half-lives calculated from k were 0.17 h for 1 and 0.25 h for 2.

Proteinase A degrades native endomorphins in two steps, similarly to carboxypeptidase Y (Table 3). In the first step, the C-terminal amide group is converted to a carboxy group, and the C-terminal amino acid is then released. This degradation mechanism is supported by the products of digestion of 3, which

Table 3
Main degradation products of 1, 2 and 3 on digestion with proteinase A

Endomorphin	t_r[a]	m/z[b]	$M_{r(calc)}$[c]	Sequence assignment	Sequence no.	$t_{1/2}$[d] (h)
1	9.8	166	165.1	Phe	4	
	15.2	465	464.3	H–Tyr–Pro–Trp–OH	1–3	
	17.9	612	611.4	H–Tyr–Pro–Trp–Phe–OH	1–4	
	17.1	611	610.4	H–Tyr–Pro–Trp–Phe–NH_2 (intact peptide)	1–4	3.5
2	9.8	166	165.1	Phe	4	
	14.9	426	425.3	H–Tyr–Pro–Phe–OH	1–3	
	17.6	573	572.4	H–Tyr–Pro–Phe–Phe–OH	1–4	
	16.6	572	571.4	H–Tyr–Pro–Phe–Phe–NH_2 (intact peptide)	1–4	1.7
3	9.8	166	165.1	Phe	4	
	14.9	426	425.3	H–Tyr–Pro–Phe–OH	1–3	
	17.6	573	572.4	H–Tyr–Pro–Phe–Phe–OH (intact peptide)	1–4	1.4

[a] HPLC retention time in min. HPLC conditions: column, Vydac 218TP54 C_{18}; gradient elution, 50% B in 20 min, see Experimental; detection, 210 nm; flow-rate, 0.8 ml/min.
[b] m/z Values for the ion $[M+H]^+$ in the mass spectra.
[c] Monoisotopic masses.
[d] Half-lives of peptides in the presence of proteinase A.

contains a C-terminal carboxy group and degrades directly to H–Tyr–Pro–Phe–OH and Phe. The remaining peptide fragments (H–Tyr–Pro–Trp–OH or H–Tyr–Pro–Phe–OH) are relatively stable. No sign of degradation products could be observed in the chromatogram after digestion for 8–10 h. The literature data indicate that proteinase A can catalyze the hydrolysis of amidated peptides [15]. Substance P (Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2) and various of its analogs degrade rapidly in the presence of proteinase A. Results concerning the stabilities of the peptides against enzymatic degradation are shown in Table 3. The total degradation of 1 requires more than 8 h, whereas 2 and 3 were no longer present after 6 h. The half-lives ($t_{1/2}$) for 1, 2 and 3 were calculated from the rate constants (k) and were found to be 3.5, 1.7 and 1.4 h, respectively.

It is difficult to explain this unusual effect of proteinase A, which belongs among the endopeptidases. On the one hand it catalyzed the conversion of the C-terminal amide group to a carboxy group, while on the other hand it behaved as an exopeptidase, splitting off one C-terminal amino acid. This behavior could not stem from impurities in the enzyme, because SDS-PAGE demonstrated its good homogenity.

4. Conclusions

HPLC combined with ESI-MS proved a useful and rapid tool for investigation of the enzymatic digestion of endomorphins. The qualitative picture of the degradation products provided by MS furnishes sufficient information to establish the possible pathways of degradation. The semiquantitative picture afforded by HPLC measurements yields useful information allowing comparisons of the stabilities of these peptides under different conditions.

In the digestion of native endomorphins, 1 and 2 with carboxypeptidase Y and proteinase A, the C-terminal amide was first converted to a C-terminal carboxy group, and the hydrolysis then led to splitting off the Trp^3–Phe^4 and Phe^3–Phe^4 bonds. The resulting tripeptides were stable against further hydrolysis with these enzymes. Carboxypeptidase A proved effective only against the peptide with a C-terminal carboxylic acid, 3. Aminopeptidase M cleaved the Pro^2–Trp^3 and Pro^2–Phe^3 linkages. The remaining N-terminal dipeptide fragment, H–Tyr–Pro–OH, was relatively stable; Tyr appeared after digestion for 5 h. The C-terminal parts, H–Trp–Phe–NH_2 or H–Phe–Phe–NH_2, hydrolyzed quickly; these dipeptides had disappeared from the reaction mixture after 1 h.

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IV

M. Spetea, K. Monory, **Cs. Tömböly**, G. Tóth, E. Tzavara, S. Benyhe, J. Hanoune and A. Borsodi

"In vitro Binding and Signalling Profile of the Novel μ Opioid Receptor Agonist Endomorphin-2 in Rat Brain Membranes."

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In Vitro Binding and Signaling Profile of the Novel μ Opioid Receptor Agonist Endomorphin 2 in Rat Brain Membranes

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The recently discovered endogenous μ receptor selective endomorphin 2 was prepared in tritiated form by a catalytic dehalogenation method resulting in a specific radioactivity of 1.98 TBq/mmol (53.4 Ci/mmol), and used for *in vitro* labelling of rat brain membranes. The binding was saturable, stereospecific and of high affinity (K_d: 0.97 and 1.12 nM based on kinetic and equilibrium binding studies, respectively). The maximal number of binding sites (B_{max}) was found to be 114.8 fmol/mg protein. [^{3}H]Endomorphin 2 was displaced by μ-receptor selective specific peptides and heterocyclic compounds with high affinity, whereas κ and δ receptor specific ligands were much less potent. The K_i values of endomorphin 1 and 2 in inhibiting [^{3}H]naloxone binding increased by 15-fold in the presence of 100 mM NaCl which indicates the agonist property of these peptides. Endomorphins stimulated [^{35}S]GTPγS binding and inhibited adenylyl cyclase activity which also provides evidence for the agonist character of endomorphins. © 1998 Academic Press

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), have recently been isolated from bovine brain. They exhibited μ agonist action on isolated guinea pig ileum, as well as 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 may be natural ligands for the morphine receptors (1).

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In the last few months a number of publications appeared showing the possible physiological relevance of endomorphin 1 and 2. These effects include electrophysiological changes in the Ca^{2+} channel currents at cellular level (2), hypotensive/vasodilator activity on the cardiovascular system (3, 4), decrease of cardiac output and total peripheral output (5), just as antinociception mediated by the spinal cord level (6). There are a few reports available on the localisation of these peptides displaying their occurrence in bovine cerebral cortex, rat medulla and spinal cord and human cortex (1, 7, 8). Very recently some functional data have also been available. Burford et al. demonstrated that the effects of endomorphin 1 are mediated through the same G proteins like morphine and DAMGO (9). Sim et al. reported endomorphin 1 evoked GTPγS binding in brain slices (10), while Hoshota et al. used both endomorphins to stimulate GTPγS binding in transfected cells (11). However, until now, no data on effects on adenylyl cyclase or direct binding data with the radiolabelled peptides have been reported.

Opioid receptors exert their biological functions by interacting with GTP binding proteins. G_i/G_o proteins, to which the opioid receptors are known to be coupled, regulate the effector molecules such as adenylyl cyclase or ion channels (12). One approach to monitor the signal transduction in the membrane preparations is the measurement of the binding of the non-hydrolysable GTP analogue, guanosine 5'-O-(γ-thio)triphosphate (GTPγS), as a function of the concentration of a given receptor ligand (13). Alternatively, the activity of adenylyl cyclase can be assessed. Both of these methods give an estimation about the ability of the ligand to activate different components of the signal transduction pathway.

In the present study, we have described the tritium labelling of endomorphin 2 with high specific radioactivity (53.4 Ci/mmol), and the binding characteristics of [^{3}H]endomorphin 2 in membrane preparations from rat brain as determined by using direct *in vitro* ligand

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binding assays. Moreover, additional data of functional approaches, i.e. effects on [^{35}S]GTPγS binding and adenylyl cyclase activity, are presented as further proof of the agonist properties of these opioid peptides.

MATERIALS AND METHODS

Materials. Endomorphin 1, endomorphin 2 and Ile^{5,6}deltorphin II (14) were synthesised in our Isotope Laboratory as previously described (15). Cyprodime was synthesised by Dr. H. Schmidhammer (Institute of Organic and Pharmaceutical Chemistry, University of Innsbruck, Innsbruck, Austria; 16). Dextrorphan and levorphanol were obtained from Hoffmann-La Roche (Nutley, NJ, USA). Naloxone hydrochloride, bovine serum albumin (BSA), captopril, bacitracin, PMSF were from Sigma Chemicals (St. Louis, MO, USA). [^{3}H]DAMGO (D-Ala^2,MePhe^4, Gly-ol^5]enkephalin, 55 Ci/mmol) and [^{3}H]*U-69,593* (5α,7α,8β-(−)-N-methyl-N[7-(1-pyrrolidinyl)-1-oxaspiro(4-5)dec-8-yl]benzacetamide, 47 Ci/mmol) were purchased from Du Pont NEN (Boston, MA). [^{3}H]Naloxone (35 Ci/mmol), [^{3}H]Ile^{5,6}deltorphin II (49.5 Ci/mmol), [^{3}H]naltrindole (46 Ci/mmol) and [^{3}H]cyprodime (31.6 Ci/mmol) were prepared in our isotope laboratory as described previously (17-20). All other reagents used in this study were of analytical grade.

Synthesis of [3', 5'-dil-Tyr^1]-endomorphin 2. The synthesis of the precursor, the tritiation procedure and the radioligand's stabilty toward enzymatic degradation is described in deatails by Tömböly et al. (21). The specific activity of tritiated endomorphin 2 was 1.98 TBq/mmol (53.4 Ci/mmol). The stability of [^{3}H]endomorphin 2 toward enzymatic degradation in rat brain membrane preparation was examined by incubating the radioligand (5 μCi) with a membrane suspension (400 μg protein) for 45 min at 25°C in a final volume of 1 ml in the presence and absence of peptidase inhibitors, as it is described in the radioligand binding assay. The chromatographic analysis showed 96.6% recovery of the tritiated endomorphin 2 in the absence of membrane fraction, while in the presence of membrane about 60% intact radioligand was recovered. When peptidase blockers were present during incubation with rat brain membranes no significant degradation occurred.

Receptor binding assays. A crude membrane fraction was isolated from Wistar rat brains according to the method previously described (22). Protein concentrations were determined by the method of Bradford (23), using bovine serum albumin as a standard.

Binding experiments with [^{3}H]endomorphin were performed in an assay buffer consisting of 50 mM Tris-HCl, 1 mg/ml BSA, 50 μg/ml bacitracin, 10 μM captopril, and 0.1 mM PMSF (pH 7.4). The final volume was 1 ml containing 300-500 μg protein. Incubations were carried out at 25°C and stopped by rapid filtration through Whatman GF/C filters, using a Brandel Cell Harvester. The filters were washed with 3–5 ml of ice-cold Tris-HCl buffer (pH 7.4). The bound radioactivity was measured in a toluene based scintillation cocktail, using a Wallac 1409 scintillation counter (η = 0.55). Non-specific binding was defined as the bound radioactivity in the presence of 10 μM unlabelled naloxone.

The time course for association of [^{3}H]endomorphin 2 was determined by incubation of the membrane preparation at 25°C for various periods of time. In the dissociation experiments, the radioligand was incubated with the membrane preparation until equilibrium and then its dissociation was initiated by the addition of an excess amount of unlabeled naloxone to give a final concentration of 10 μM. Saturation binding experiments were performed by incubating the membranes with increasing concentrations of [^{3}H]endomorphin 2 from 0.01 to 6 nM concentration. Equilibrium binding studies were carried out by incubating protein with 0.5 nM [^{3}H]endomorphin 2 in the presence of different concentrations of unlabeled ligands. All assays were carried out in duplicate and repeated at least three times; the given values are the mean ± SEM. Kinetic data were analysed according to the method described by Weiland and Molinoff (24). Competition inhibition constants (K_i) were calculated with the LIGAND program (version 2.3.22) utilising a non-linear least squares fitting algorithm (25). Hill coefficient values were obtained by analysing heterologous competition binding data with the GraFit 3.0 programme (26).

Membrane preparation for functional assays. In the functional assays, low pH treated membrane preparations were used as it was described by Selley et al. (27).

[^{35}S]GTPγS binding. Experiments were carried out in 50 mM Tris-HCl buffer containing 1 mM EGTA and 3 mM MgCl in a final volume of 1 ml. Assay tubes, containing 10 μg of protein, 30 μM GDP, 1 nM - 10 μM opioid ligands and 0.05 nM [^{35}S]GTPγS were incubated for 1 hr, at 30°C. For positive control morphine was utilised. Non-stimulated activity was measured in the absence of tested compounds, while non-specific binding was measured in the presence of 100 μM non-labelled GTPγS. The incubation was terminated by filtrating the samples through Whatman GF/B glass fibre filters. Filters were washed three times with ice-cold 50 mM Tris-HCl buffer (pH 7.4) in a Millipore filtration instrument. Filters were then dried, and radioactivity was measured in a Wallac 1409 scintillation counter (Turku, Finland) using a toluene based scintillation cocktail. Stimulation is given as percent of the specific binding. Data were calculated from three independent experiments performed in triplicates.

Measurement of adenylyl cyclase activity. This assay was conducted as described earlier (28). Membranes (15-40 μg protein per assay) were preincubated with the agonists or with vehicle for 10 min at room temperature. The antagonist CTAP was added 10 min before the addition of the agonists. All assays were run in triplicates.

RESULTS AND DISCUSSION

In the present paper we report a comprehensive study including binding characteristics of endomorphins in normal and tritiated form and functional properties of these ligands in rat brain membrane preparations.

The recent discovery of endomorphin 1 and 2 by Zadina et al. (1) can be considered as 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 more than two decades after the description of the endogenous ligands for the δ and κ receptors (enkephalins and dynorphins; 29, 30), there was no acceptable candidate for the μ receptors.

A series of opioid radioligands with different receptor specificity were used to confirm the selectivity of endomorphin 1 and 2. The equilibrium inhibition constants (K_i values) of endomorphins were compared to K_i values of radioligands calculated from homologous displacement studies (Table I). Endomorphin 1 and 2 competed for the [^{3}H]naloxone binding sites with high affinities, while in the presence of 100 mM NaCl, the inhibitory constants increased by 15-fold. This suggests the pure agonist property of both peptides (31). Somewhat better affinities were observed with the μ specific radioligands, such as the peptide derivative [^{3}H]DAMGO and the heterocyclic [^{3}H]cyprodime. The highly δ selective [^{3}H]Ile^{5,6}deltorphin II (20), and the

TABLE I

Competition Abilities of Endomorphin Peptides in a Set of Type-Selective Opioid Radioligand Binding Assays in Rat Brain Membranes

		K_i (nM)		
			Heterologous	
Ligand	Selectivity	Homologous	Endomorphin 1	Endomorphin 2
[^{3}H]naloxone	(general antagonist)	1.7[a] ± 0.26	9.5[a] ± 2.5	11.6[a] ± 1.3
		0.8[b] ± 0.02	126[b] ± 26	152[b] ± 50
[^{3}H]DAMGO	(μ agonist)	2.5 ± 1.1	4.2 ± 1.3	5.3 ± 1.5
[^{3}H]cyprodime	(μ antagonist)	13.1 ± 3.2	10.2 ± 2.8	13.2 ± 2.2
[^{3}H]Ile^{5,6}deltorphin II	(δ agonist)	0.7 ± 0.3	1540 ± 310	>10000
[^{3}H]naltrindole	(δ antagonist)	0.8 ± 0.2	>10000	4760 ± 690
[^{3}H]U-69,593	(κ agonist)	2.3 ± 0.6	>10000	>10000

Note. Rat brain membranes were incubated with the radioligands in the presence of different endomorphine concentrations (10^{-12}-10^{-5} M) in duplicate. Incubation and filtering conditions were as follows: 60 min, 0 °C for [^{3}H]naloxone (22); 45 min, 35 °C for [^{3}H]DAMGO (40); 40 min, 25 °C for [^{3}H]cyprodime; 45 min, 35 °C for [^{3}H]Ile^{5,6}deltorphin II (20); 90 min, 25 °C for [^{3}H]naltrindole (41); 30 min, 30 °C for [^{3}H]U-69,593 (42). K_i values for homologous competition experiments were determined by LIGAND analysis. Heterologous displacement curves were first analysed by GraFit programme using the 'four parameter logistic' fitting option (26). IC_{50} values calculated by GraFit were then converted into K_i values on the basis of the Cheng-Prusoff equation. Data are mean values ± S.E.M. of 2-4 independent determinations.

[a] Without NaCl.

[b] In the presence of 100 mM NaCl.

heterocyclic [^{3}H]naltrindole (19) were used to assess the binding to δ receptor. The affinity of endomorphins for this site was very low. Neither endomorphin 1, nor endomorphin 2 competed for the κ receptor binding sites that have been labelled by [^{3}H]U-69,593 in a measurable range.

Zadina and colleagues found the affinity of these ligands in the subnanomolar range measured by the μ receptor specific peptides [^{3}H]DAMGO. According to our results, the affinities of endomorphin 1 and 2 for [^{3}H]DAMGO binding sites seems to be a little lower than those reported by Zadina and colleagues, though still in the low nanomolar range (4.2 and 5.3 nM for endomorphin 1 and 2, respectively). However, our data is in a good agreement with Sakaguchi et al. (32), who measured Phe^4-morphiceptin (endomorphin 2) against [^{3}H]DAMGO and got a K_i value of 6.6 nM.

We have provided evidence for this specificity coming from direct radioligand binding experiments too. Endomorphin 2 was prepared in radiolabelled form by a dehalotritiation method through [3',5'-diI-Tyr^1]endomorphin 2 (21). This precursor was tritiated in the presence of Pd/$BaSO_4$. The end-product showed a high, 1.98 TBq/mmol (53.4 Ci/mmol) specific radioactivity. The radioligand showed satisfactory stability as determined by HPLC analysis (data not shown) and degradation under binding assay condition was totally prevented by the use of proper peptidase inhibitors.

Kinetic studies revealed that [^{3}H]endomorphin 2 binding to rat brain membranes reaches a steady state in 40 min incubation at 25°C. The association (k_{+1}) and dissociation rate constant (k_{-1}) were calculated and found to be 0.088 ± 0.008 $min^{-1} \cdot nM^{-1}$ and 0.085 ± 0.001 min^{-1}, respectively. The "kinetic" dissociation constant (K_d) value of 0.97 nM was resolved from the kinetic studies.

The equilibrium binding of [^{3}H]endomorphin 2 to rat brain membranes was saturable and showed high affinity (Fig. 1). Scatchard analysis of the saturation binding data revealed that a single class of binding sites was labelled (Fig. 1, inset). Equilibrium constant (K_d) and the maximal number of binding sites (B_{max}) were determined and found to be 1.12 ± 0.25 nM and

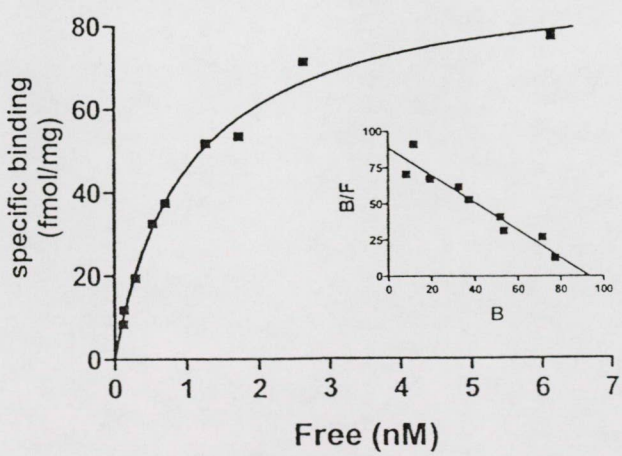

FIG. 1. Saturation isotherms of [^{3}H]endomorphin 2 binding to rat brain membranes. Increasing concentrations of [^{3}H]endomorphin 2 were incubated with membranes in the presence and absence of 10 μM naloxone for 45 min at 25°C for estimating non-specific binding. The Scatchard plot of obtained binding data (inset) revealed only one class of binding sites.

TABLE II

Inhibition of [^{3}H]Endomorphin 2 Binding with Various Type-Selective Opioid Ligands in Rat Brain Membranes

Competitor ligand	Type-selectivity	K_i (nM)	Slope (n_H)
Naloxone	general antagonist	0.80 ± 0.27	0.93 ± 0.02
Endomorphin 1	μ agonist	3.7 ± 0.56	0.93 ± 0.03
Endomorphin 2	μ agonist	1.88 ± 0.15	0.94 ± 0.08
DAMGO	μ agonist	2.15 ± 0.50	0.88 ± 0.11
Cyprodime	μ agonist	7.89 ± 0.43	0.68 ± 0.06
(−)Levorphanol	μ agonist	0.45 ± 0.03	0.90 ± 0.06
(+)Dextrorphan	inactive enantiomer	973 ± 30	1.04 ± 0.23
U-69,593	κ agonist	480 ± 96	0.87 ± 0.16
Ile^{5,6}deltorphin II	δ agonist	2723 ± 103	0.74 ± 0.19

Note. Membranes were incubated with 0.5 nM [^{3}H]endomorphin 2 in the presence of increasing concentrations of unlabelled opioid ligands for 45 min at 25°C. K_i values were determined by LIGAND analysis assuming homologous binding site (one-site fit). Slopes of the competition curves were further analysed by the programme GraFit (26) to obtain Hill coefficient values (n_H). Each value represents the mean ± S.E.M. of 3-4 experiments.

114.80 ± 19.57 fmol/mg protein, respectively. The K_d values for [^{3}H]endomorphin 2 determined from saturation experiments were found to be in good agreement with those derived from the kinetic studies (see above).

The obtained B_{max} value was in agreement with the values reported for μ receptor density for other μ receptor selective peptide radioligands, such as [^{3}H]TAPP (33), [^{3}H]DAMGO (34), [^{3}H]PL-017 (35), and [^{3}H]dermorphin (36). The binding was stereospecific as found by displacements of biologically potent (levorphanol) and inactive (dextrorphan) enantiomers of a chemically identical ligand, where more than two orders of magnitude difference was observed in their K_i values (Table II).

Competition binding assays with various unlabelled type-specific opioid ligands for the [^{3}H]endomorphin 2 sites in rat brain membrane were carried out to evaluate their abilities to inhibit the binding. These studies revealed the following rank order of potencies in displacing [^{3}H]endomorphin 2 binding: $\mu \gg \kappa > \delta$. The inhibition constants (K_i values) are shown in Table II. The μ receptor specificity of the radioligand was undoubtedly proved by the competition experiments. Competition curves for [^{3}H]endomorphin 2 were best fitted by a one-site model. Accordingly, Hill-coefficients were close to the unity, indicating again that [^{3}H]endomorphin 2 labels interacts with a single set of binding sites.

Functional assays were performed on striatal membranes which are known to be enriched in μ opioid receptors (37). The membrane preparations were carried out using the low pH treatment method which is known to eliminate the G_s proteins leaving active the G_i proteins, thus, allows to increase maximal inhibition of adenylyl cyclase by agonists for G_i coupled receptors (27).

Endomorphin 1 and 2 stimulated [^{35}S]GTPγS binding in rat striatal membranes is a dose-dependent manner. The maximal effectes were about 270% of the non-stimulated level for endomorphins, while it was

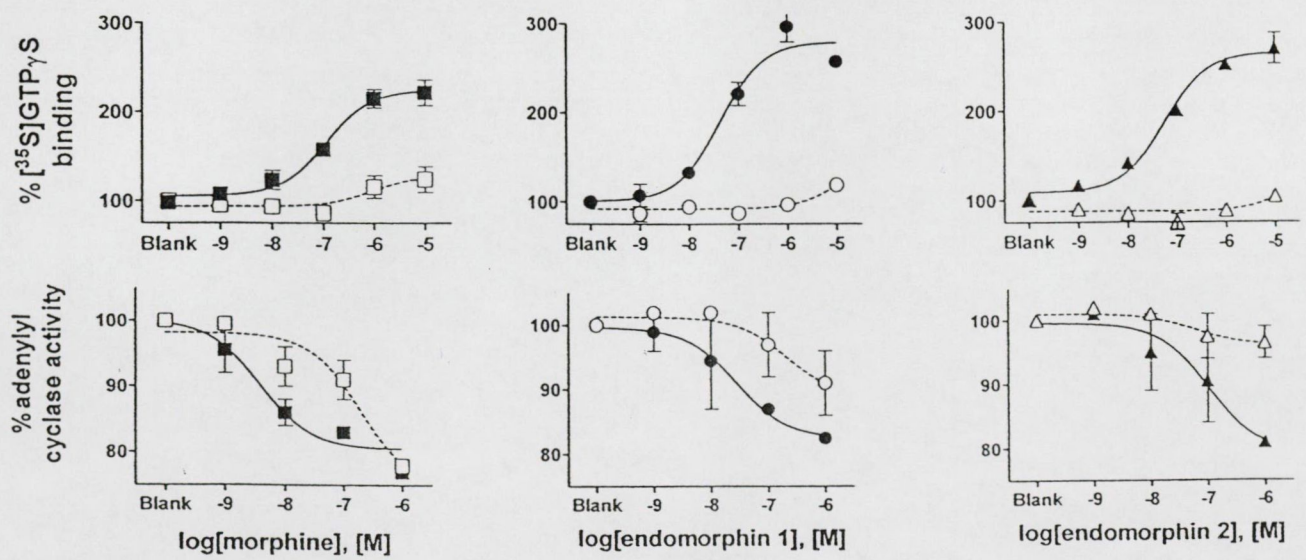

FIG. 2. Functional assays carried out on rat striatal membranes. Top: Stimulation of [^{35}S]GTPγS binding by different concentrations of morphine (■), endomorphin 1 (●) and endomorphin 2 (▲) in the absence (filled symbols) and presence (empty symbols) of 1 μM CTAP. Incubations were carried out for 60 min. at 30°C. Non-specific binding was 44%, non-stimulated [^{35}S]GTPγS binding was 109.6 ± 29.5 fmol/mg protein. Bottom: Inhibition of adenylyl cyclase activity by different concentrations of morphine (■), endomorphin 1 (●) and endomorphin 2 (▲) in the absence (filled symbols) and presence (empty symbols) of 10 μM CTAP. Incubations were carried out for 10 min. at 37°C. Points represent means ± S.E.M. from two or three independent experiments carried out in triplicates.

TABLE III

ED_{50} and Maximal Effect Values Achieved in Functional Assays in Rat Striatal Membranes

	[^{35}S]GTPγS binding				Adenylyl cyclase activity			
	+ CTAP		+ CTAP		+ CTAP		+ CTAP	
	ED_{50} (nM)		Maximal effect (%)		ED_{50} (nM)		Maximal effect (%)	
Morphine	112	897	224	128	4	196	80	74
Endomorphin 1	44	>10,000	279	no effect	28	208	83	89
Endomorphin 2	57	>10,000	265	no effect	103	>10,000	79	no effect

Note. Ability of morphine and endomorphins to stimulate [^{35}S]GTPγS binding (left) and inhibit adenylyl cyclase activity (right) in rat striatal membranes measured in the absence and presence of the μ opioid receptor antagonist peptide, CTAP. ED_{50} values. Data are in nM, and represent the means of 3 independent experiments. Maximal stimulation given in percentage of the non-stimulated level. Data were fitted with Graph Pad Prism 2.01.

lower by 20-25% for morphine (Fig. 2, top, Table III). Both endomorphin 1 and 2 showed somewhat better ED_{50} values than morphine (Table III). The reversibility of these effects have been also tested by using a μ opioid receptor specific antagonist. For this purpose, the somatostatin analogue peptide, CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2), was used at 1 μM concentration (38). CTAP at the used concentration completely abolished the effects of endomorphins (Fig. 2, top, empty symbols).

Inhibition of adenylyl cyclase has been also assessed in rat striatum. The basal level of adenylyl cyclase activity (100%) was dose-dependently inhibited in the presence of endomorphin 1 or endomorphin 2 by 17-20% (Fig. 2, bottom). These maximal inhibition values were identical to that of morphine (Table III) and they are in good agreement with those reported in the literature (39). The ED_{50} values were calculated from these curves and found to be 28 nM for endomorphin 1, 103 nM for endomorphin 2 and 4 nM for morphine. The inhibitory effects of these ligands were reversed by CTAP at a concentration of 10 μM, as it was indicated by the significant shift to the right in the dose-response curves (Fig. 2, bottom, empty symbols). This antagonist was not able to inhibit the morphine effect above the micromolar concentration of the opiate agonist morphine.

In conclusion, for the unambiguous approval for μ opioid receptor specificity of endomorphin 1 and 2, *in vitro* ligand binding as well as functional assays were utilised. Both peptides showed high degree of μ receptor selectivity when competing with type specific radioligands labelling the different opioid receptor populations in rat brain membranes. Endomorphin 2 was synthesised in tritiated form with high, 53.4 Ci/mmol, specific radioactivity for direct labelling. The binding of [^{3}H]endomorphin 2 was found to be saturable and stereospecific. A single class of high affinity binding site was detected, from which the [^{3}H]endomorphin 2 was readily displaced by μ receptor selective peptides and heterocyclic compounds. The affinities of δ and κ specific ligands were several orders of magnitude lower. Both peptides stimulated [^{35}S]GTPγS binding and inhibited adenylyl cyclase in rat striatal membranes as did morphine, the prototypic μ agonist. The effects of endomorphins in [^{35}S]GTPγS binding and adenylyl cyclase assays were inhibited by the selective μ receptor antagonist, CTAP. Agonist character and μ receptor specificity of endomorphin 1 and 2 was undoubtedly proved by *in vitro* binding and functional approaches. [^{3}H]endomorphin 2 is a unique μ receptor specific radioligand of high affinity, which can be used for further biochemical and pharmacological investigations of the opioid receptor system.

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"Specific Activation of the μ Opioid Receptor (MOR) by Endomorphin 1 and Endomorphin 2."

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Specific activation of the μ opioid receptor (MOR) by endomorphin 1 and endomorphin 2

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Abstract

The recently discovered endomorphin 1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin 2 (Tyr-Pro-Phe-Phe-NH_2) were investigated with respect to their direct receptor-binding properties, and to their ability to activate G proteins and to inhibit adenylyl cyclase in both cellular and animal models. Both tetrapeptides activated G proteins and inhibited adenylyl cyclase activity in membrane preparations from cells stably expressing the μ opioid receptor, an effect reversed by the μ receptor antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2), but they had no influence on cells stably expressing the δ opioid receptor. To further establish the selectivity of these peptides for the μ opioid receptor, brain preparations of mice lacking the μ opioid receptor gene were used to study their binding and signalling properties. Endomorphin 2, tritiated by a dehalotritiation method resulting in a specific radioactivity of 1.98 TBq/mmol (53.4 Ci/mmol), labelled the brain membranes of wild-type mice with a K_d value of 1.77 nM and a B_{max} of 63.33 fmol/mg protein. In membranes of mice lacking the μreceptor gene, no binding was observed, and both endomorphins failed to stimulate [^{35}S]guanosine-5′-O-(3-thio)triphosphate ([^{35}S]GTPγS) binding and to inhibit adenylyl cyclase. These data show that endomorphins are capable of activating G proteins and inhibiting adenylyl cyclase activity, and all these effects are mediated by the μ opioid receptors.

Introduction

It is more than two decades since the three endogenous opioid peptide families (endorphins, enkephalins and dynorphins) have been identified and characterized (for a review, see Akil *et al.*, 1984). Localization of these peptides and of their binding sites has been extensively studied by autoradiographical and immunohistochemical methods all over the central nervous system. Pharmacological approaches have shown the existence of three types of opioid receptors, μ, δ and κ (Martin *et al.*, 1976; Lord *et al.*, 1977). Although selectivity of the enkephalins for the δ receptor (Hughes *et al.*, 1975) and dynorphins for the κ receptor (Goldstein *et al.*, 1979) was demonstrated, no endogenous ligand has been attributed to the μ receptor until very recently, although morphine and related compounds, which are clinically useful and subject to abuse, are believed to act primarily through the μ receptors (Matthes *et al.*, 1996).

Zadina and colleagues isolated two peptides, named endomorphin 1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin 2 (Tyr-Pro-Phe-Phe-NH_2), first from bovine (Zadina *et al.*, 1997) and later from human cortex (Hackler *et al.*, 1997). These peptides differ from the previously known endogenous opioid ligands in their N-terminal sequence (Tyr-Pro versus Tyr-Gly), length and C-terminal amidation. Their isolation and description was considered as a major breakthrough in opioid research. The high affinity and good selectivity of these peptides for the μ receptor were indirectly shown by displacements of [^{3}H]-DAMGO, i.e. of [^{3}H]-[D-Ala^2,(N-Me)Phe^4,Gly^5-ol]enkephalin, and by the facts that they were found to be co-localized with μ receptors (Zadina *et al.*, 1997), and capable of inducing some of the physiological responses classically attributed to morphine. Thus, they show vasodilator properties in rat (Champion *et al.*, 1997a,b; Czapla *et al.*, 1998), rabbit (Champion *et al.*, 1997c) and mice (Champion *et al.*, 1998a), although there is an apparent controversy about the mechanism of these effects (Rialas *et al.*, 1998, Champion & Kadowitz, 1998b; Champion et al., 1998c). Both peptides decrease the electrically evoked muscle contractions in GPI preparations (Tonini *et al.*, 1998) by inhibiting acetylcholine release from the myenteric plexus (Nishiwaki *et al.*, 1998, Yokotani & Osumi, 1998). They are effective in spinal antinociceptive tests (Stone *et al.*, 1997), and have orexigenic and anxiolythic effects (Asakawa *et al.*, 1998).

Recently, we have shown that endomorphin 1 and 2 activate G proteins and inhibit adenylyl cyclase in rat striatal membranes (Spetea *et al.*, 1998). In the present study, we intended to prove the exclusive μ opioid receptor specificity of these peptides. For this purpose, we have used Chinese hamster ovary (CHO) cells stably transfected with either the μ or the δ opioid receptor, and brain preparations of wild type or μ knockout mice, the latter lacking completely μ opioid binding sites (Matthes *et al.*, 1996). By both functional assays [measuring the binding of the non-hydrolysable GTP analogue guanosine-5′-O-(3-thio)triphosphate (GTPγS) to G proteins and adenylyl cyclase activity], and direct *in vitro* radioligand binding assays using tritiated endomorphin 2, we demonstrate that

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endomorphins are inactive on cell and tissue preparations lacking μ receptors.

Materials and methods

Materials

ATP, cAMP, phosphocreatine, creatine phosphokinase, DADLE (D-Ala^2,D-Leu^5-enkephalin), forskolin (FSK), and phenylmethylsulphonyl fluoride (PMSF) were from Sigma (St. Louis, MO, USA). [^{32}P]ATP (30 Ci/mmol) and 8-[^{3}H]cAMP (30 Ci/mmol) were from Amersham (Arlington Heights, IL, USA). Ready Safe liquid scintillant was purchased from Beckman (Fullerton, CA, USA). Cell culture reagents and antibiotics were from Gibco Life Sciences (Grand Island, NY, USA). All other chemicals were of the highest purity commercially available. Morphine–HCl was purchased either from SANOFI (Montpelier, France) or ICN Alkaloida (Tiszavasvári, Hungary). CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) was a generous gift from NIDA (Rockville, MD, USA).

Animals

Mutant mice, deficient in the μ opioid receptor, were generated in CNRS UPR 9050, Illkirch, France (Matthes *et al.*, 1996). Animals used in this study were of hybrid B6/129 genetic background.

Cell lines

CHO cell line stably transfected with μ opioid receptor gene, 3.5 pmol/mg membrane protein (CHOμ; Capeyrou *et al.*, 1997) was a generous gift of Dr L. Emorine (CNRS UPR 9062, Toulouse, France). CHO cells stably expressing the δ opioid receptor, 4.3 pmol/mg membrane protein (CHOδ), have been described earlier (Befort *et al.*, 1996).

Synthesis of tritiated endomorphin 2

3′,5′-^{3}H-Tyr^1-endomorphin 2 (Tyr-Pro-Phe-Phe-NH_2) was prepared by catalytic dehalogenation of diiodinated precursor ([3′,5′-I]Tyr-Pro-Phe-Phe-NH_2) using Pd/$BaSO_4$ catalyst and $^3H_2$ gas in tritium manifold (Tömböly *et al.*, 1999). The crude tritiated peptide was purified by thin layer chromatography on Kieselgel 60F254 plate (BuOH : AcOH : H_2O, 4 : 1 : 1). Purity was checked by high-performance liquid chromatography (HPLC) [on LiChrospherR 100 RP-18 (5 mm, Merck, Darmstadt, Germany): eluent acetonitrile/0.1% TFA in water gradient of 20–35% organic component in 25 min (flow rate, 1 mL/min); Rt, 10.51 min] and was found to be >95%. Specific radioactivity was 1.98 TBq/mmol (53.4 Ci/mmol).

Cell culture and membrane preparations

CHOμ and CHOδ cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Sciences, Grand Island, NY, USA) and in α-minimum essential medium (MEM, Gibco Life Sciences), respectively. Both media were supplemented with 10% foetal calf serum, 2 mM glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin, 25 mg/mL fungizone and 0.5 mg/mL geneticin. Cells were maintained in culture at 37 °C in a 5% CO_2 atmosphere. Membranes were prepared from subconfluent cultures. Cells were rinsed three times with 10 mL phosphate-buffered saline (PBS) and removed with (in mM): Tris–HCl, 20, pH 7.6; EGTA, 0.1; EDTA, 0.5; β-mercaptoethanol, 5; PMSF buffer, 0.5, and homogenized for 15 s with a polytron homogenizer in an ice-bath, followed by centrifugation at 500 *g* for 5 min. The supernatant was centrifuged at 17 000 *g* for 20 min. The resulting pellet was resuspended in the above buffer and centrifuged again. The final pellet was reconstituted in a small volume of the following buffer (in mM): Tris–HCl, 20, pH 7.6; EGTA, 1; EDTA, 0.5; β-mercaptoethanol, 2.5; PMSF, 0.25.

[^{35}S]GTPγS binding

Tubes containing 5 (cell membranes) or 10 (brain membranes) μg of protein, 30 μM GDP, 10^{-9}–10^{-5} M opioid ligands and 0.05 nM [^{35}S]GTPγS, all in 50 mM Tris–HCl buffer containing 1 mM EGTA and 3 mM $MgCl_2$ in a final volume of 1 mL were incubated for 1 h, at 30 °C. For positive control, morphine was utilized. Non-stimulated activity was measured in the absence of tested compounds, non-specific binding was measured in the presence of 10 μM unlabelled GTPγS. CTAP was added 10 min before the addition of the protein. The incubation was started by the addition of the [^{35}S]GTPγS and was terminated by filtrating the samples through Whatman GF/B glass fibre filters. Filters were washed three times with ice-cold 50 mM Tris–HCl buffer (pH 7.4) in a Millipore filtration instrument, then dried. Bound radioactivity was measured in a Wallac scintillation counter (Turku, Finland) using a toluene-based scintillation cocktail. Stimulation is given as per cent of the specific binding. Data were calculated from three independent experiments performed in triplicate and evaluated with Prism 2.01 from GraphPad (San Diego, CA, USA).

Measurement of adenylyl cyclase activity

This assay was performed on CHO cell membranes and striatal membranes from wild-type and MOR knockout animals. Mice were killed by cervical dislocation and brains rapidly removed. Brains were dissected at 4 °C to remove the striatum. Striata were homogenized in 10 vol. of an ice-cold homogenization buffer consisting of (in mM): Tris–HCl, 20, pH 8; EDTA, 1; DTT, 0.5; PMSF, 0.5. Homogenates were centrifuged at 15 000 *g* at 4 °C for 30 min, pellets were resuspended in the same buffer and stored at −80 °C until use. Cell (15–40 μg protein per assay) or brain (40–80 μg protein per assay) membranes were preincubated with the agonists or with vehicle for 10 min at room temperature. The antagonist CTAP was added 10 min before the addition of the agonists. The formation of cAMP was conducted at 35 °C for 10 min in 60 μL of an assay medium with the following composition (in mM): Tris, 50, pH 7.6; $MgCl_2$, 5; GTP, 10 μM; cAMP, 1; ATP, 1, containing 10^6 c.p.m. [^{32}P]ATP, in a regenerating system consisting of 5 mM creatine phosphate and 250 μg/mL creatine kinase. The reaction was started with the addition of the substrate and was stopped by 500 mM HCl. Reaction mixtures were neutralized with 1.5 M imidazol followed by separation of the cAMP from the ATP on alumina columns. The amount of [^{32}P]cAMP formed was measured and corrected for the recovery of added [^{3}H]cAMP. All assays were run in triplicate. Data were evaluated with Prism 2.01 from GraphPad.

Measurement of cAMP accumulation

CHOμ or CHOδ cells were plated at 300 000 cells per well in 12-well plates and grown in the appropriate medium (see Cell culture and membrane preparation). After 24 h, the medium was replaced by serum-free medium. Twenty-four hours later, the medium was removed and the cells were rinsed with PBS, and the reaction performed in 1 mL PBS in the presence of 600 μM 3-isobuthyl-1-methylxanthine (IBMX). The cells were incubated in the presence of the agonists at the indicated concentrations (ranging from 0.5 pM to 1 μM) for 15 min before the addition of 0.5 μM of forskolin. After another 15 min, the medium was removed and the reaction was stopped by the addition of 1 mL cold ethanol. The cAMP was extracted at room temperature for 3 h. The amount recovered was then determined by RIA (kit purchased from Immunotech, USA).

according to the instructions of the manufacturer. Each determination was performed in duplicate and the resulting data were then evaluated with Prism 2.01 from GraphPad.

Receptor-binding assays

Crude brain membrane preparations for the *in vitro* binding studies were carried out according to the method described by Simon *et al.* (1986). Protein concentration was determined by the method of Bradford (1976).

The frozen mice brain membranes were thawed and separated from the Tris–HCl/sucrose medium by centrifugation at 40 000*g* for 20 min at 4 °C. Pellets were resuspended to a protein concentration of 0.2–0.3 mg/mL in an assay buffer consisting of 50 mM Tris–HCl, 1 mg/mL bovine serum albumin (BSA), 50 mg/mL bacitracin, 10 mM captopril, 30 mg/mL bestatin and 0.1 mM PMSF (pH 7.4). Aliquots of mice membrane were incubated with radioligand for 45 min at 25 °C, at conditions which were shown to be appropriate in kinetic experiments in a final volume of 1 mL. The reactions were terminated by rapid filtration under vacuum through Whatman glass fibre filters (GF/C), followed by washing with 3 × 5 mL of ice-cold 50 mM Tris–HCl buffer (pH 7.4) using a Brandel Cell Harvester (Cambridge, MA, USA). Filters were dried and the bound radioactivity was measured in scintillation vials containing 5 mL of toluene-based scintillation cocktail using a Wallac Scintillation Counter. Non-specific binding was determined in the presence of 10 μM naloxone. Saturation binding experiments were performed by incubating the membranes with increasing concentrations of [^{3}H]endomorphin 2 (0.05–7.5 nM). All assays were performed in duplicate and repeated three times. The given values represent the means (± SEM). Data were evaluated with Prism 2.01 from GraphPad.

Results

Different concentrations of endomorphin 1 and endomorphin 2 were used to stimulate [^{35}S]GTPγS binding and to inhibit adenylyl cyclase activity in two different membrane preparations: CHO cells transfected with either the human μ opioid receptor (Capeyrou *et al.*, 1997) or with the mouse δ opioid receptor (Befort *et al.*, 1996). The adenylyl cyclase activity was determined on cell membrane preparations and by the accumulation of cyclic AMP (cAMP) in whole cells (see Materials and methods). In membranes of CHOμ cells (Fig. 1), both peptides stimulated [^{35}S]GTPγS binding and inhibited cAMP synthesis.

In CHOμ cell membranes the maximal stimulation produced by endomorphins on [^{35}S]GTPγS binding was ~2.5-fold of the non-stimulated value (Fig. 1a). The same effect was observed with morphine, which was used as a reference compound. This stimulation was completely reversed by CTAP, the somatostatin analogue μ opioid receptor-specific antagonist (Kramer *et al.*, 1989).

The ability of endomorphins to inhibit adenylyl cyclase activity was also tested in CHOμ cell membrane preparations. Adenylyl cyclase activity measurements, in purified membranes using [^{32}P]ATP as the substrate over a 10-min period, showed 12–18% inhibition below the control level (data not shown). The inhibition of the adenylyl cyclase activity produced by endomorphins, although reproducible, was rather low. This phenomenon is usually observed when the agonist is acting through G_i-coupled receptors, a type of G protein that is known to be activated by opioid receptors (Standifer & Pasternak, 1997). A more accurate determination was achieved by measuring the cAMP accumulation in whole cells in the presence of 0.5 μM forskolin and the phosphodiesterase inhibitor IBMX. Thus, the maximal values of inhibition of cAMP accumulation were 53% for endomorphin 1 and 54% for endomorphin 2; DAMGO, a synthetic μ receptor-specific agonist peptide, inhibited cAMP accumulation by 54%. The EC_{50} values were 0.24 nM for endomorphin 1, 0.10 nM for endomorphin 2 and 0.21 nM for DAMGO (Fig. 1b).

When examining CHO cells transfected with the mouse δ opioid receptor, no stimulation of [^{35}S]GTPγS binding was detected as an effect of endomorphins (Fig. 2a). However, Ile^{5,6}deltorphin II, a potent δ agonist peptide (Nevin *et al.*, 1994), caused a twofold stimulation over the control level. Moreover, in CHOδ cells, significantly less inhibition of cAMP accumulation was detected in the presence of endomorphins, i.e. ~20–25% inhibition (Fig. 2b). By contrast, DADLE, a potent δ receptor agonist, produced an inhibition of cAMP accumulation of 69% below the control level.

Endomorphin 2 was prepared in tritiated form with a specific radioactivity of 53.4 Ci/mmol (Tömböly *et al.*, 1999), and its binding properties were first determined in membrane preparations of CHOμ cells. The binding of [^{3}H]endomorphin 2 was saturable and of high affinity (Fig. 3a). Scatchard analysis of data was best fitted with a single binding site model (Fig. 3b). The equilibrium dissociation constant (K_d) value and maximal number of binding sites (B_{max}) were determined and found to be 2.29 ± 0.39 nM and 490.12 ± 25.33 fmol/mg protein, respectively. Non-specific binding of [^{3}H]endomorphin 2 in membrane preparations of CHOμ cells was under 30% of total binding at a radioligand concentration equal to the K_d value. In membrane preparations of CHOδ cells, no binding could be observed at any of the radioligand concentrations used (Fig. 3c).

In the next set of experiments, the effects of endomorphins in brain membranes of mice lacking the μ opioid receptor gene were examined (Matthes *et al.*, 1996). For analysing the effects of endomorphins on [^{35}S]GTPγS binding in mutant and wild-type mice, we utilized the same type of membrane preparations as for the *in vitro* binding studies. In membranes of wild-type mice all the ligands tested (endomorphin 1 and 2, and the δ receptor-specific compound, Ile^{5,6}deltorphin II) gave a 30–40% maximal stimulation of [^{35}S]GTPγS binding over the basal (non-stimulated) level (Fig. 4a). By contrast, endomorphins did not show any stimulation of [^{35}S]GTPγS binding to G proteins in membranes from mice lacking μ opioid receptors, while the δ receptor-selective Ile^{5,6}deltorphin II was fully active.

We have further characterized the effects of endomorphins on adenylyl cyclase activity in striatal membranes of mice lacking the μ opioid receptor gene and their wild-type littermates (Fig. 4b). In striatal membranes of wild-type mice, both endomorphin 1 and 2 inhibited adenylyl cyclase activity. The maximal values of inhibition were 13% for endomorphin 1 and 16% for endomorphin 2, respectively. In brain membranes of mutant mice, no change in the level of adenylyl cyclase activity was achieved in the presence of endomorphins. The δ opioid receptor agonist, DADLE, had a similar effect in inhibiting adenylyl cyclase activity for both membranes of wild-type or mutant animals, resulting in maximal inhibition values of 17 and 18%, respectively.

Binding experiments with [^{3}H]endomorphin 2 in brain membrane preparations of wild-type and mutant mice have also been carried out. The equilibrium binding of [^{3}H]endomorphin 2 was saturable and showed high affinity (Fig. 5a). Scatchard analysis of data revealed that a single set of binding sites was labelled (Fig. 5b). The equilibrium dissociation constant (K_d) value and maximal number of binding sites (B_{max}) were determined and found to be 1.77 ± 0.64 nM and 63.33 ± 11.18 fmol/mg protein, respectively. In brain membrane preparations of the homozygous μ receptor-deficient mutant mice, no binding could be observed at any of the radioligand concentrations used (Fig. 5c).

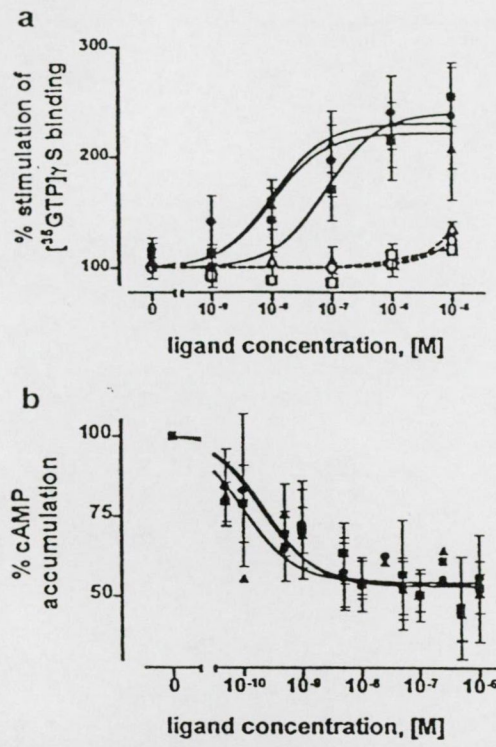

FIG. 1. Functional assays carried out on CHOμ cells. (a) Stimulation of [^{35}S]GTPγS binding by different concentrations of endomorphin 1 (●), endomorphin 2 (▲) and morphine (■) in the absence (filled symbols) and presence (empty symbols) of 1 μM CTAP. Incubations were carried out for 60 min at 30 °C. Non-specific binding was 64%, non-stimulated [^{35}S]GTPγS binding was 43.23 ± 4.33 fmol/mg protein. Points represent means ± SEM from three separate experiments performed in triplicate. (b) Inhibition of cAMP accumulation by different concentrations of endomorphin 1 (●), endomorphin 2 (▲) and DAMGO (■). Control values were 374.36 ± 79.88 fmol cAMP/μg protein. Points represent means ± SEM from three separate experiments performed in duplicate.

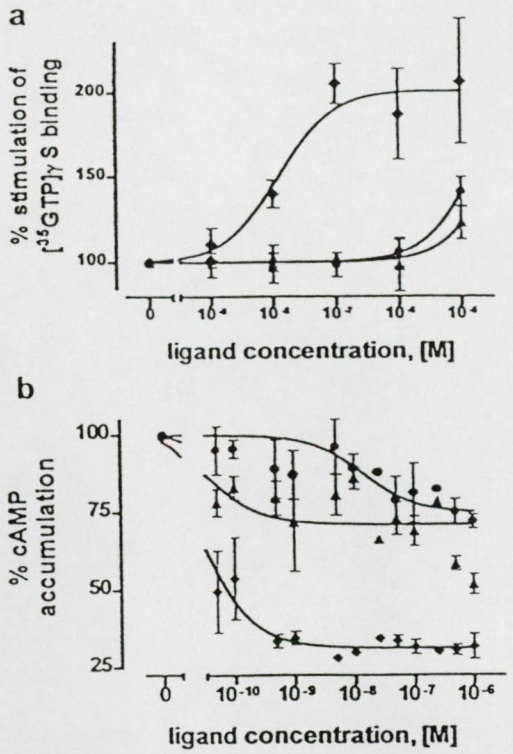

FIG. 2. Functional assays carried out on CHOδ cells. (a) Stimulation of [^{35}S]GTPγS binding by different concentrations of endomorphin 1 (●), endomorphin 2 (▲) and the δ receptor agonist Ile^{5,6}deltorphin II (◆). Incubations were carried out for 60 min at 30 °C. Non-specific binding was 64%, non-stimulated [^{35}S]GTPγS binding was 32.86 ± 7.91 fmol/mg protein. Points represent means ± SEM from three separate experiments performed in triplicate. (b) Inhibition of cAMP accumulation by different concentrations of endomorphin 1 (●), endomorphin 2 (▲) and the δ receptor agonist DADLE (◆). Control values were 361.34 ± 72.65 fmol cAMP/μg protein. Points represent means ± SEM from three separate experiments.

Discussion

The ability of different concentrations of the tetrapeptides endomorphin 1 and 2 to generate an intracellular response was first tested in membrane preparations of CHO cells transfected with either the human μ or the mouse δ opioid receptor.

In CHOμ cells endomorphins activated G proteins as indicated by the stimulation of the binding of [^{35}S]GTPγS, a non-hydrolysable GTP analogue, to membrane preparations in a dose-dependent manner (Fig. 1a) in accordance with the recent literature (Alt *et al.*, 1998; Harrison *et al.*, 1998, Hosohata *et al.*, 1998). The stimulation was strong and significant, ~2.5-fold of the non-stimulated value. Morphine, the prototypic μ opioid agonist, and used here as the reference compound, had the same effect in these experiments, although endomorphins had slightly better EC_{50} values than morphine; the EC_{50} values were 10.78 nM for endomorphin 1, 10.64 nM for endomorphin 2 and 80.60 nM for morphine. However, in CHOδ cells no stimulation of [^{35}S]GTPγS binding could be observed by endomorphin 1 and 2, while Ile^{5,6}deltorphin II, a potent δ opioid agonist peptide, produced a twofold stimulation over the non-stimulated level with an EC_{50} value of 12.98 nM (Fig. 2a).

As a next step, we examined the effects of endomorphins on the activity of adenylyl cyclase in the membrane preparations of the transfected cells. In these assays both peptides inhibited cyclase activity in preparations of CHOμ cells, though the maximal inhibition values observed were only 12–18%. The inhibition was limited, but similar to those previously reported in the literature. Thus, an inhibition of 12–17% has been reported for morphine in brain preparations (Duman *et al.*, 1988; Nijssen *et al.*, 1992; Carter & Medzihradsky, 1993). In order to confirm these data, cAMP accumulation in cell cultures, in the presence of 0.5 μM forskolin and endomorphins at various concentrations, was determined. In CHOμ cells both tetrapeptides inhibited the accumulation of cyclic AMP (Fig. 1b). The maximal values of inhibition were strong and significant, more than 50% for both endomorphins and DAMGO, a known μ opioid receptor-specific peptide. Furthermore, the EC_{50} values were in the subnanomolar range. In membranes of CHOδ cells, however, endomorphins inhibited much less of cAMP accumulation. In contrast, the δ opioid agonist peptide DADLE was an effective inhibitor of cAMP production in that system reaching a maximal inhibition of ~70% (Fig. 2b) with an EC_{50} value as low as 0.03 nM. The above data clearly demonstrate that endomorphins functionally modulate adenylyl cyclase activity in CHO cells expressing the μ opioid receptor. By contrast, cells carrying the δ opioid receptor are not capable to respond upon endomorphin exposure (Fig. 2), indicating that endomorphins are not likely to bind to δ opioid receptors.

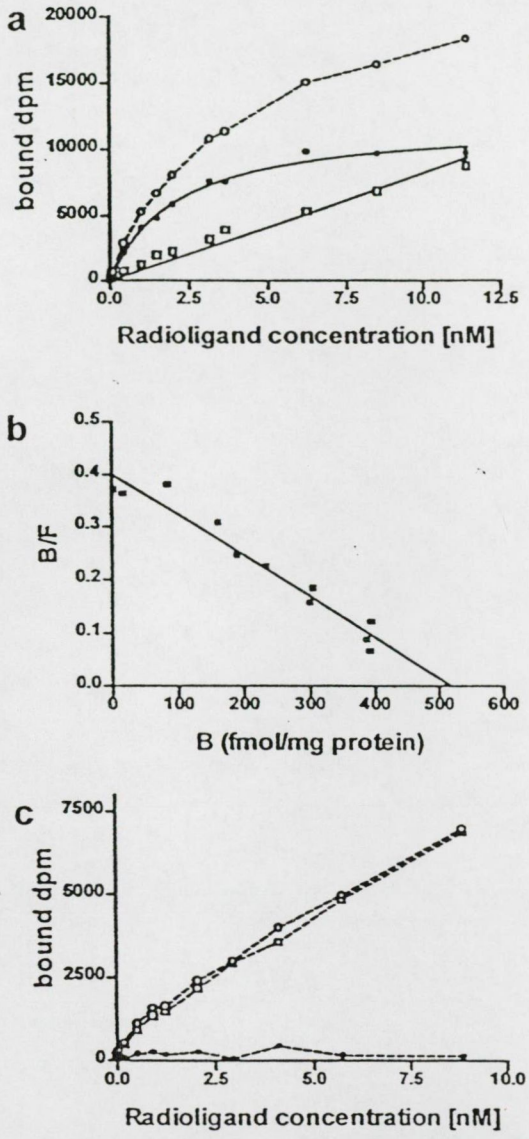

FIG. 3. (a) Saturation binding of [^{3}H]endomorphin 2 to membranes of CHOμ cells. Plots represent total (○), non-specific (□) and specific (●) binding in dpm. Incubations were carried out in 50 mM Tris buffer (pH 7.4) for 45 min at 25 °C. (b) Scatchard transformation of the data. (c) Saturation binding of [^{3}H]endomorphin 2 to membranes of CHOδ cells. Plots represent total (○), non-specific (□) and specific (●) binding in dpm. These are representative experiments which were replicated three times.

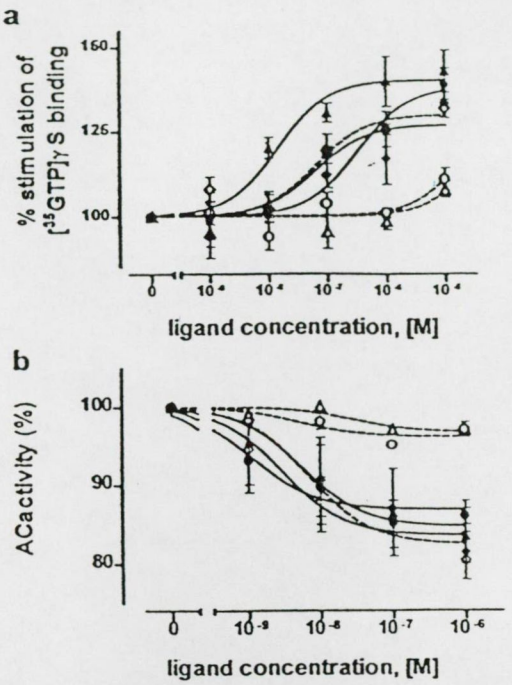

FIG. 4. Functional assays carried out on brain membranes of wild-type and mutant mice, those lacking the μ opioid receptor. (a) Stimulation of [^{35}S]GTPγS binding by different concentrations of endomorphin 1 (●), endomorphin 2 (▲) and the δ receptor agonist Ile^{5,6}deltorphin II (◆) in membranes of wild-type (filled symbols) and MOR knockout mice (empty symbols). Incubations were carried out for 60 min at 30 °C. Non-specific binding was 37 and 39%, non-stimulated [^{35}S]GTPγS binding was 91.65 ± 13.77 and 72.90 ± 7.87 fmol/mg protein for the wild-type and the knockout animals, respectively. Points represent means ± SEM from three separate experiments performed in triplicate. (b) Inhibition of adenylyl cyclase activity by different concentrations of endomorphin 1 (●), endomorphin 2 (▲) and the δ receptor agonist DADLE (◆) in membranes of wild-type (filled symbols) and MOR knockout mice (empty symbols). Incubations were carried out for 10 min at 37 °C. Points represent means ± SEM from three separate experiments performed in triplicate.

The more pronounced effect of endomorphins in cAMP assay compared with adenylyl cyclase activity measurements may be due to different regulatory factors present in the whole cells, but lost during the membrane preparations. In CHO cells the presence of AC type VI and VII has been reported (Varga *et al.*, 1998). The Ca^{2+}-inhibitable AC VI isoform (Yoshimura & Cooper, 1992; Katsushika *et al.*, 1992) is regulated by different entities in the cell: $G_i\alpha$, $G\beta\gamma$, protein kinase C (PKC) and protein kinase A (PKA, Taussig *et al.*, 1994; Thomas & Hoffman, 1996; Iwami *et al.*, 1995; Lai *et al.*, 1997; Bayewitch *et al.*, 1998). Among the effectors potentially involved in AC VI activity regulation, Ca^{2+} amounts were proved to be modulated by endomorphins through inhibition of high-threshold Ca^{2+}-channels (Mima *et al.*, 1997; Higashida *et al.*, 1998). The marked inhibition of cAMP accumulation in the cells may result from integrated co-incident signals at the cyclase level.

Considering the results presented in Figs 1 and 2, we hypothesized that endomorphins do not bind to δ opioid receptors. In order to further investigate this, endomorphin 2 was prepared in a radiolabelled form using a dehalotritiating method (Tömböly *et al.*, 1999). The resulting ligand had high specific radioactivity (1.98 TBq/mmol or 53.4 Ci/mmol). Membranes of CHOμ and CHOδ cells were incubated with different concentrations of [^{3}H] endomorphin 2 in order to determine the binding parameters. In CHOμ cells, [^{3}H] endomorphin 2 labelled a single set of binding sites with a K_d value of 2.29 ± 0.39 nM and a B_{max} of 490.12 ± 25.33 fmol/mg protein (Fig. 3a and b). However, when CHOδ cells were studied, no specific binding could be obtained (Fig. 3c). This means that only CHO cells transfected with μ but not δ opioid receptors carry the binding sites to which endomorphins are able to couple.

In order to further investigate whether endomorphins act only through the μ opioid receptor, or could activate other receptor types as well, μ receptor-deficient animals were used. The use of transgenic mice compared with transfected cells served as an *in vivo* validation of our experimental approach too. Matthes *et al.* (1996) showed that MOR knockout animals are deprived of μ opioid binding sites

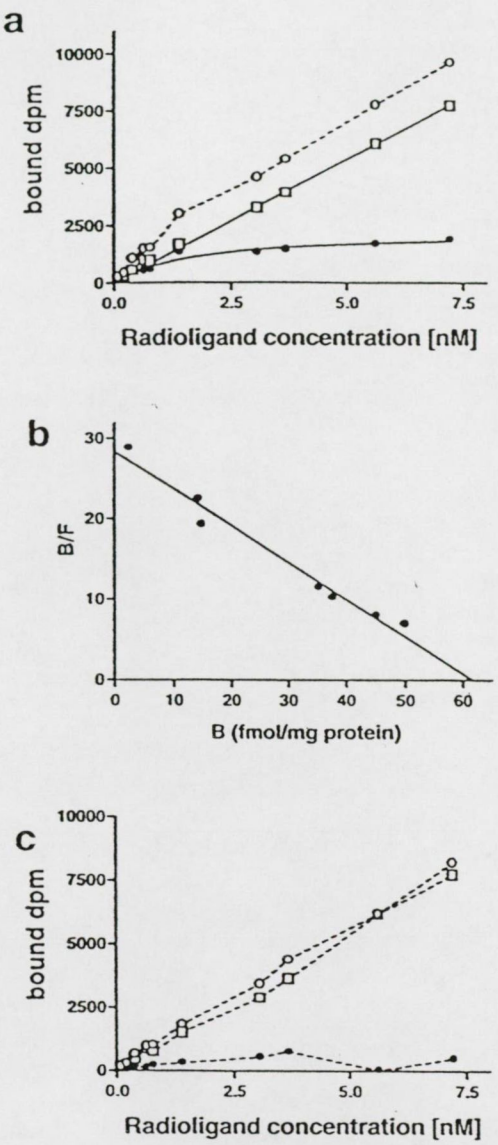

FIG. 5. (a) Saturation binding of [^{3}H]endomorphin 2 to brain membranes of wild-type mice. Plots represent total (○), non-specific (□) and specific (●) binding in dpm. Incubations were carried out in 50 mM Tris buffer (pH 7.4) for 45 min at 25 °C. (b) Scatchard transformation of the data. (c) Saturation binding of [^{3}H]endomorphin 2 to brain membranes of μ knockout mice. Plots represent total (○), non-specific (□) and specific (●) binding in dpm. These are representative experiments which were replicated three times.

without showing alterations of either δ or κ binding sites, or of the expression pattern of the endogenous opioid peptide genes proenkephalin, prodynorphin and proopiomelanocortin. Our experimental approaches included direct radioligand binding assays with tritiated endomorphin 2 and functional experiments ([^{35}S]GTPγS binding and adenylyl cyclase activity measurements) with endomorphin 1 and 2 using wild-type and mutant mice.

The obtained K_d and B_{max} values from direct radioligand binding experiments with [^{3}H]endomorphin 2 in wild-type animals (1.77 nM and 63 fmol/mg protein, respectively) are in agreement with those reported by Matthes *et al.* (1996) using the μ receptor-selective radioligand, [^{3}H]DAMGO (K_d, 1.34 nM; B_{max}, 99 fmol/mg protein), and is also consistent with our recent finding that [^{3}H]endomorphin 2 labels a receptor population of 114.8 fmol/mg protein with a K_d value of 1.12 nM in rat brain membrane preparations (Spetea *et al.*, 1998). However, in μ receptor-deficient mutant mice, no binding was detected at any of the used radioligand concentrations (Fig. 5). These observations show that endomorphin 2 specifically labels μ opioid receptor sites and thus directly demonstrate the high specificity of this compound for the μ receptor as suggested by Zadina *et al.* (1997).

The functional assays further confirmed the results found in the radioligand binding studies. That is, endomorphins stimulated [^{35}S]GTPγS binding and inhibited adenylyl cyclase activity in brain preparations of wild-type mice, but only the δ receptor-selective ligands Ile^{5,6}deltorphin II and DADLE were able to produce these changes in preparations of MOR knockout mice (Fig. 4). These results undoubtedly demonstrate that endomorphins are unable to initiate intracellular events in the absence of μ opioid receptors.

It is interesting to note that, in the case of wild-type mice, there is a considerable difference between EC_{50} values obtained in the [MS Serif ^{35}S]GTPγS binding assay for endomorphin 1 and 2, a phenomenon which was not seen in membranes of CHOμ cells (present study) or rat striata (Spetea *et al.*, 1998). There are a few possible explanations for this. The one amino acid sequence difference in the two tetrapeptides may account for some difference in the conformation of the molecules, which in turn might result in a different degree of activation of the mouse μ opioid receptor. Although rat and mouse μ opioid receptors differ only in 10 amino acids, this small difference may be responsible for subtle changes in the binding or the G protein-activating properties. Another possible explanation is the different resistance to peptidases of endomorphin 1 and 2, as suggested by Stone *et al.* (1997). In this case, we should suggest too, that the expression pattern and/or level of peptidases is not the same in rats and mice. Concerning the physical effects evoked by endomorphins, most authors report similar effects by both peptides, although in some cases there are differences in the reported responses. For example, in rat dorsal horn neurons endomorphin 1 and endomorphin 2 have distinct inhibitory effects (Chapman *et al.*, 1997); endomorphin 2 seems to have selective effects on noxious stimuli whereas endomorphin 1 is non-selective. Spinal actions of endomorphins were similar in mice (Stone *et al.*, 1997), though a longer pretreatment was necessary to observe acute tolerance with endomorphin 1 than endomorphin 2 (90 versus 30 min, respectively). Finally, endomorphin 1 was slightly better than endomorphin 2 in evoking an orexigenic effect (Asakawa *et al.*, 1998). The above data show that although the structural elements involved are not known yet, the opioid system is able to distinguish between these closely related structures.

It is worth mentioning that, despite the numerous publications that appeared in the last 2 years dealing with endomorphins, the amount of data available is not similar to the bulk of data about the other endogenous opioids, e.g. β-endorphin or enkephalins. The high affinity, the excellent specificity and the different physiological responses evoked all converge to the conclusion that these peptides are ligands of the μ opioid receptor. But are they really 'the' endogenous μ opioids? Or are there more new ones yet to come? These problems might not be answered before the precursor molecule of endomorphins is characterized.

In conclusion, we have demonstrated that endomorphin 1 and 2 inhibit cAMP formation and increase the binding of the GTP analogue GTPγS to G proteins in CHOμ cells and brains of wild-type mice through binding to and activation of μ opioid receptors, specifically and exclusively.

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Abbreviations

BSA, bovine serum albumin; CHO, Chinese hamster ovary; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; DADLE, D-Ala^2,D-Leu^5-enkephalin; DAMGO, [D-Ala^2,(N-Me)Phe^4,Gly^5-ol]enkephalin; DMEM, Dulbecco's modified Eagle's medium; FSK, forskolin; GTPγS, guanosine-5′-O-(3-thio)triphosphate; HPLC, high-performance liquid chromatography; IBMX, 3-isobuthyl-1-methylxanthine; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PMSF, phenylmethylsulphonyl fluoride.

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VI

G. Tóth, **Cs. Tömböly**, A. Péter, D. Biyashev, A. Borsodi, A. Rónai and R. Przewłocki

"New Endomorphin Analogues: Design, Synthesis and Biological Properties."

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New Endomorphin Analogues: Design, Synthesis and Biological Properties

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Introduction

Endomorphin 1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin 2 (Tyr-Pro-Phe-Phe-NH_2) isolated from the bovine and human brain have been suggested as being endogenous ligands for μ opioid receptor [1]. These peptides have high affinity and selectivity for μ opioid receptor in direct and indirect radioreceptor assay in rat and mouse brain and recombinant μ opioid receptors. Endomorphins mediate analgesia that can be blocked by naloxone and other μ antagonists. [2]

This presentation deals with the design and synthesis of new analogues of endomorphins using conformationally constrained amino acids (β-MePhe, Dmt), examination of their *in vitro* opioid activity profile in binding and GPI and MVD assays and studies on the structure-opioid activity relationship.

Results and Discussion

β-MePhe and Dmt were synthesized in our laboratory by literature methods [3,4] to obtain erythro- and threo-β-MePhe and Dmt in racemic form. The optically pure forms of both amino acids were obtained by enzymatic resolution using N-trifluoroacetyl derivatives of the amino acids and carboxypeptidase A. Peptides were prepared by the SPPS method with incorporation of racemic erythro or threo-β-MePhe or L-Dmt in the peptides. Diastereomeric peptides were separated by RP-HPLC. The configuration of β-MePhe in the peptides was determined after hydrolysis using the GITC reagent.

In vitro opioid activities of the new endomorphin analogues were determined in guinea pig ileum (GPI) and mouse vas deferens (MVD) assays and their μ and δ receptor affinities were measured in radioreceptor assays based on displacement of μ ([^{3}H]endomorphin 2) and δ ([^{3}H]Ile^{5,6}-deltorphin II) selective radioligands from rat brain membrane binding sites (Table 1). The effect of methylation of the β carbon of the Phe side chain on the biological properties of endomorphins depends on the chirality of the C_α and C_β of β-MePhe. In all cases, endomorphins with L-β-MePhe (2S,3S or 2S,3R) had higher affinity than the D-β-MePhe containing isomers. Endomorphins with (2S,3S)β-MePhe in position 4 are 4-5 times more active compared to the parent peptides. (2S,3S)β-MePhe^4-endomorphin 2 is the

most selective analogue for the μ opioid receptor. Endomorphin 2 analogues containing β-MePhe isomers in position 4 have very similar trends in GPI and MVD assay as in the binding assay. These analogues are agonists. Endomorphins with β-MePhe in position 3 are less selective compared to the parent peptides.

Binding affinities of the new Dmt[1]-endomorphin analogues were very high on μ and δ receptors, too. The results show the importance of methyl groups on the message part of the endomorphins. In the GPI and MVD assays both endomorphin analogues are agonists with more than ten times lower IC_{50} values compared to the parent peptides. According to the results, the ligands bind to the μ opioid receptors in GPI and MVD as well.

Table 1. *In vitro opioid activities of endomorphin analogues*

	Binding assay			GPI	MVD
	$K_{i\_}$ (nM)	$K_{i\delta}$ (nM)	$K_{i\delta}$ / $K_{i\_}$	IC_{50} (nM)	IC_{50} (nM)
Tyr-Pro-Trp-Phe-NH_2	1.62	6390	3944	10.1	61.6
Tyr-Pro-Phe-Phe-NH_2	4.00	2650	663	9.22	22.1
Tyr-Pro-Trp-(2S,3S)β-MePhe-NH_2	0.47	567	1206		
Tyr-Pro-Trp-(2R,3R)β-MePhe-NH_2	43.6	1460	33.5		
Tyr-Pro-Trp-(2S,3R)β-MePhe-NH_2	23.4	4630	198		
Tyr-Pro-Trp-(2R,3S)β-MePhe-NH_2	47.0	745	15.9		
Tyr-Pro-(2S,3S)β-MePhe-Phe-NH_2	73.1	179	2.45		
Tyr-Pro-(2S,3R)β-MePhe-Phe-NH_2	35.0	>10000	>286		
Tyr-Pro-Phe-(2S,3S)β-MePhe-NH_2	0.97	6360	6557	3.46	9.15
Tyr-Pro-Phe-(2R,3Rβ)-MePhe-NH_2	127 μ	5180	40.8 μ	817	>10000
Tyr-Pro-Phe-(2S,3R)β-MePhe-NH_2	23.4	4900	209	89.9	236
Tyr-Pro-Phe-(2R,3S)β-MePhe-NH_2	47.7	>10000	>210	589	>10000
Dmt-Pro-Trp-Phe-NH_2	0.0049	12.0	2449	0.81	0.54
Dmt-Pro-Phe-Phe-NH_2	0.039	93.6	2400	0.70	0.89

Acknowledgments

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VII

R. Przewłocki, D. Łabuz, J. Mika, B. Przewłocka, **Cs. Tömböly** and G. Tóth

"Pain Inhibition by Endomorphins."

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Pain Inhibition by Endomorphins

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ABSTRACT: **Spinal analgesic effects of endomorphin-1 and endomorphin-2 were studied during acute, inflammatory, and neuropathic pain in rats chronically implanted with intrathecal cannulas. Endomorphin-1 and endomorphin-2 (2.5–10 μg i.t.), as well as their analogues, increased the tail-flick and the paw pressure latencies. In a model of inflammatory pain, the formalin-induced behavior was attenuated by endomorphins; however, the effect studied was not dose-dependent and was less pronounced in comparison with that evoked by morphine. On the other hand, in rats with a sciatic nerve injury (crush), endomorphins antagonized allodynia in a dose-dependent manner, whereas morphine was found to be ineffective in a similar dose range. Endomorphins also exhibited an antinociceptive potency in rats tolerant to morphine. In conclusion, our results show a powerful analgesic action of endomorphins at the spinal level. The most interesting finding is a strong effect of endomorphins in neuropathic pain, which opens up a possibility of using these compounds in pain therapy.**

INTRODUCTION

Two potent endogenous opioid peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), have been recently isolated from bovine and human brain.[1,2] These peptides differ in their amino acid sequences from other known endogenous opioid peptides in which the Tyr residue is followed by Gly. Endomorphin sequences are related to the family of previously discovered opioid peptides containing Tyr-Pro residues, such as β-casomorphins, morphiceptin, and hemorphin. Although the latter peptides display opioid-like activity, they have not been found in the brain. On the other hand, other structurally related peptides, such as Tyr-MIF-1, which have been found in the brain, show a certain affinity for opioid receptors, but display antagonistic as well as agonistic activity.[3,4]

The discovery of endomorphins opens up new possibilities in opioid research. Both peptides label the μ-opioid receptor with a high affinity and selectivity.[2] These endomorphins have been shown to induce analgesia via μ-opioid receptors.[2,5–7] Further, immunoreactivity of endomorphin-2 was found in the central nervous system,

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in regions associated with nociception and rich in μ-opioid receptors, such as the spinal cord and thalamus. This peptide may be synthesized in the ganglia of primary sensory neurons and then transported to superficial layers of the dorsal horn of the spinal cord, as has already been demonstrated in the rat and monkey.[2,8–10] Endomorphin-2 is localized mainly in primary sensory afferents. In addition, endomorphin-2 immunoreactivity was found colocalized in a subset of substance P and μ-opiate receptor-containing fibers in the spinal cord. This indicates that endomorphins may be major endogenous opioid ligands for pre- and postsynaptic spinal μ-opioid receptors, and could be a critical regulator of pain perception.

We demonstrate here that endomorphins inhibit nociceptive transmission in the spinal cord through μ-opioid receptors. However, the mode of activity of those peptides differs significantly from that of morphine. First, endomorphins appear to be less potent than morphine in antagonizing inflammatory pain. Second, in contrast to morphine, both endomorphins show strong analgesic activity in neuropathic pain. These effects of endomorphins contrast with previous observations showing that the analgesic efficacy of opioids is increased in inflammation and reduced in neuropathic pain. Futhermore, endomorphin-1 displayed clear analgesic activity in morphine-tolerant rats, indicating a lack of cross-tolerance with morphine. Endomorphins display the highest sensitivity and selectivity toward the μ-opioid receptor compared with other endogenous opioid peptides, and their spinal action is mediated through the μ-opioid receptor. However, μ-opioid receptor subtypes and independent intracellular signal transduction pathways may be involved in the differential effects of endomorphins and morphine. Alternatively, endomorphins may interact with unknown non-opioid receptors, resulting in modulation of their opioid actions.

ENDOMORPHIN EFFECTS ON NOCICEPTION

Current studies describe the analgesic activity of the endomorphin-1 and -2. Both of these peptides showed a potent, dose- and time-dependent antinociceptive effect after intraventricular and intrathecal (i.t.) injection in mice.[2,5]

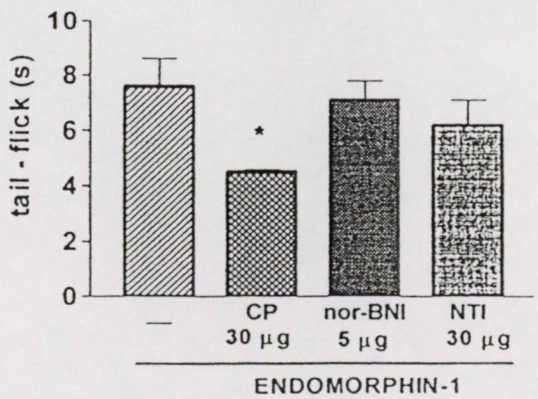

FIGURE 1. Effect of opioid antagonists cyprodime (CP), nor-binaltorphimine (nor-BNI), and naltrindole (NTI) on endomorphin-1 analgesia.

Our present study shows a spinal analgesic activity of endomorphins in rats. Both endomorphin-1 and -2 exerted a potent dose- and time-dependent antinociceptive effect after i.t. injection to rats via a chronically implanted catheter. The antinociceptive effect of endomorphins was observed after acute thermal and mechanical stimuli. The response to thermal stimuli was long-lasting, whereas that to mechanical stimuli was substantially shorter.[6]

Endomorphin-1 (2.5–10 μg i.t.) dose-dependently increased the tail-flick latency to radiant heat. The maximal effect was observed at 15 and 30 min after injection, but was significant up to 120 min after doses of 5 and 10 μg. The paw pressure latency was significantly increased at 15–30 min after all the doses used, but at 60 min was significant only at a dose of 10 μg.

Endomorphin-2 (2.5–10 μg i.t.) also dose-dependently increased the tail-flick latency. About a 50% increase was observed after a dose of 10 μg that lasted up to 120 min. The paw pressure latency was significant only up to 30 min after administration of endomorphin-2. The antinociceptive effect of these endomorphins in acute pain models in rats was weaker than that of DAMGO, but comparable to the effect of morphine. The antinociceptive effect of endomorphins was antagonized by the selective μ receptor antagonist cyprodime (30 μg i.t), but not by the κ receptor antagonist norbinaltorphimine or the δ receptor antagonist naltrindole (FIG. 1).

A spinal antinociceptive effect of endomorphins was recently reported in mice using a tail-flick test.[7] However, in the latter study, endomorphins produced short-term antinociception, the maximum effect developing at 2 min post-injection and disappearing completely at 10–15 min post-injection. In contrast, in our present study, a prolonged antinociceptive effect was found in rats. The maximum was reached after approximately 15 min, and that effect lasted longer than 2 h. The reason for such a profound difference in spinal antinociceptive activity in mice and rats is presently unknown; it can be speculated, however, that it may be due to differences in the metabolism of these peptides in the two species. On the other hand, the original study[2] as well as a more recent one[5] reported strong and prolonged spinal activity in the mouse tail-flick test. Stone *et al.*[7] showed that the peptides endomorphin-1 and endomorphin-2 administered spinally were equipotent when tested in a warm-water immersion tail-flick assay in mice. Interestingly, in our present study, endomorphin-2 was distinctly less potent in comparison with endomorphin-1 in both the the tail-flick and the paw pressure tests. This observation is in agreement with the results obtained recently in mice, which also showed that endomorphin-2 was significantly less active than endomorphin-1.[2,5]

The antinociceptive effect of endomorphins at both the spinal and the supraspinal level was antagonized by the non-selective opioid antagonist naloxone and the μ-opioid receptor selective antagonists β-FNA and cyprodime,[11] as well as, to a lesser extent, by the μ_1-receptor antagonist naloxonazine.[5] Neither κ- nor δ-selective antagonists were effective. This is consistent with the observations that endomorphins are highly selective ligands for the μ-opioid receptor in binding assays and that β-FNA reversed their analgesic actions in mice.[2] Further, these peptides may be released in response to painful and traumatic stimuli and thus act as endogenous analgesics. This hypothesis is further supported by a recent study that shows the presence of these peptides in nociceptive pathways at both the spinal and the supraspinal level of the neuroaxis.[8]

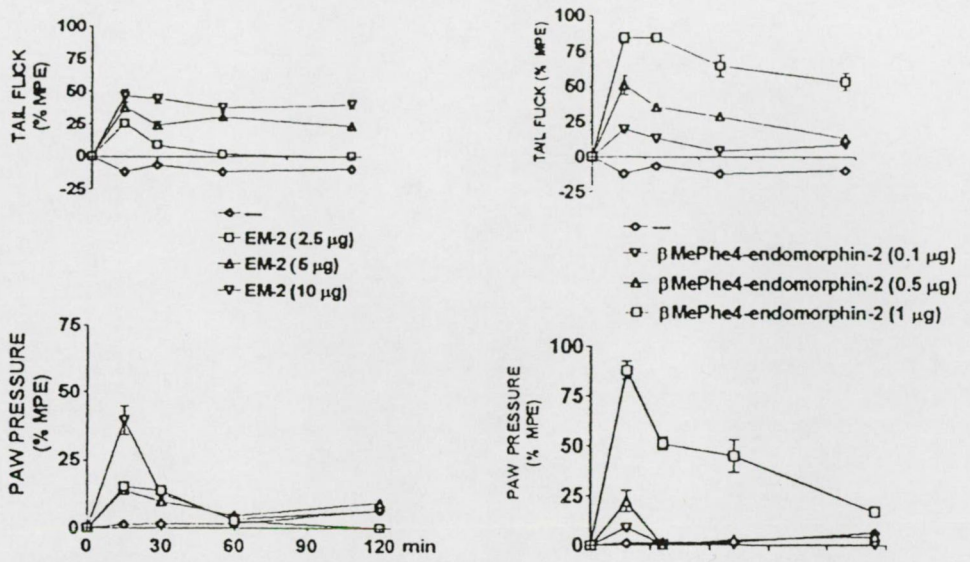

FIGURE 2. Effect of endomorphin-2 (EM-2) and its analogue Tyr-Pro-Phe(2S,3S) βMePheNH$_2$ on nociceptive threshold.

We conducted further studies into the antinociceptive effect of the novel analogues of endomorphins; βMePhe4–endomorphin-1 and -2, methylated at the position 4. Endomorphin-1 and endomorphin-2 and their analogues were synthesized by a solid-phase method on a 4-methyl-benzhydrylamine resin by means of a Boc chemistry.[12] Both endomorphin analogues showed slightly enhanced affinity toward the μ sites and increased μ/κ selectivity, as well as μ/δ selectivity in case of endomorphin-2.

βMePhe4–Endomorphin-1, an analogue of endomorphin-1, also increased the tail-flick latency dose-dependently (0.1–10 μg i.t.). The analgesic effect was comparable with that exerted by the parent peptide. The increase in the paw pressure latency was only slightly stronger and lasted longer than that evoked by the parent peptide endomorphin-1.

βMePhe4–Endomorphin-2, an analogue of endomorphin-2, showed a much higher antinociceptive activity than the parent peptide. It increased the tail-flick latency dose-dependently (0.1–1 μg i.t.) to radiant heat. The maximal effect was observed at 15 and 30 min after injection, but was significant up to 120 min after a dose of 1 μg. The paw pressure latency was slightly increased at 15 min after doses of 0.1 and 0.5 μg, but it was very pronounced and lasted up to 120 min only after a dose of 1 μg (FIG. 2).

The analogue appears to be about 20 times more potent in the tail-flick and paw pressure test when compared to the parent peptide and represents an interesting endomorphin analogue for future study. Further, these results suggest an importance of β-methylation of the endomorphin in increasing μ-site selectivity and affinity of the peptide.

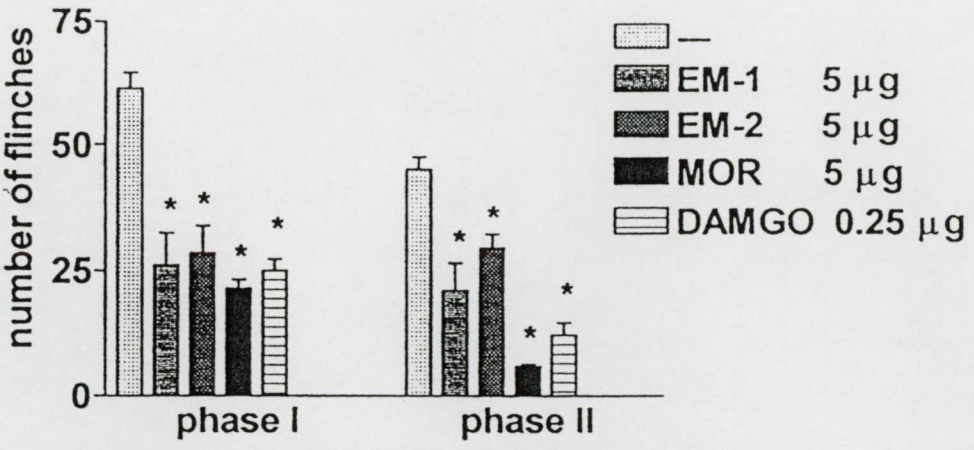

FIGURE 3. The antinociceptive effect of endomorphin-1 (EM-1), endomorphin-2 (EM-2), DAMGO, and morphine (MOR) in a formalin test.

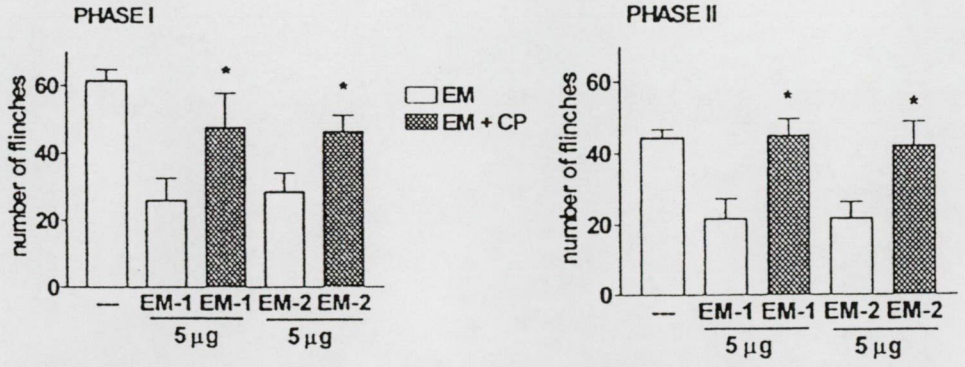

FIGURE 4. Effect of cyprodime (CP) on endomorphin analgesia in formalin model.

EFFECTS OF ENDOMORPHIN ON INFLAMMATORY PAIN

We studied the effects of endomorphin-1 and endomorphin-2, administered i.t., in acute inflammatory pain, which was induced by intraplantar injection of formalin.[6] The pain-related behavior (a number of formalin-induced flinching episodes) was significantly reduced by endomorphin-1 in both phases of the formalin-evoked pain (FIG. 3).

However, both endomorphins appear to be less effective in antagonizing the formalin-induced pain than in inhibiting the nociceptive responses evoked by acute thermal or pressure stimuli. In contrast, the μ-opioid agonists DAMGO and morphine strongly inhibited the pain-related behavior induced by formalin (FIG. 3). Futher, the effect evoked by endomorphin-2 was less pronounced than that of endomorphin-1. The antinociceptive effect of endomorphins was antagonized by the μ-opioid antagonist cyprodime (30 μg i.t.) (FIG. 4), indicating that the effect was mediated via μ-opioid receptors.

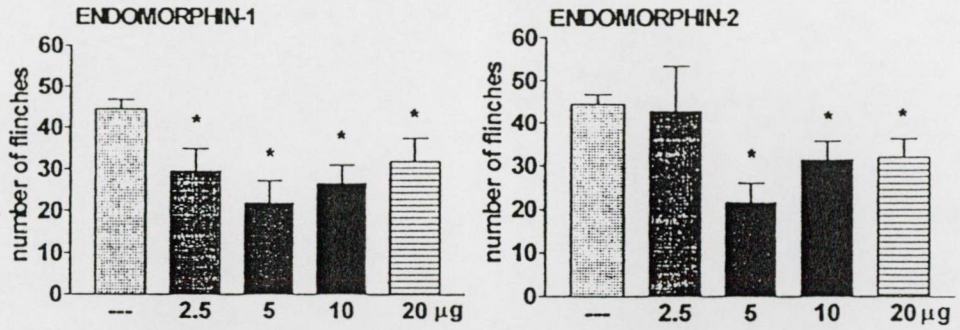

FIGURE 5. Effect of endomorphins on formalin-induced pain.

Interestingly, these results suggest that endomorphins appear to be less active and shorter-acting than other μ-opioid receptor agonists, such as morphine, in this model of experimental inflammatory pain. Further, the analgesic effect of both endomorphins was not dose-dependent (FIG. 5).

It has been previously demonstrated that the analgesic efficacy of opioids is increased in inflammation. The effect appears to be related to decreased activity of spinal cholecystokinin upon inflammation—a system that seems to modulate opiate-induced analgesia. Endomorphins showed lower analgesic potency than morphine and DAMGO. This was particularly evident in the second phase of formalin-evoked inflammation. In addition, activity of both peptides was not dose related. In fact, higher doses of endomorphins, such as 10 and 20 μg, evoked lower effects than the dose of 5 μg. Thus, the present study demonstrates that the analgesic effects of endomorphins decrease in inflammatory pain, an effect that is significantly different from that of morphine.

EFFECTS OF ENDOMORPHIN ON NEUROPATHIC PAIN

Neuropathic pain has been assumed to be resistant to treatment with opiates. It is well established that morphine and μ-receptor-selective opiates are poor analgesics in neuropathic pain in humans and in models of neuropathic pain in animals. It has been demonstrated that the effect of morphine is markedly reduced by peripheral axotomy, spinal cord injury[13,14] or neuropathic pain in rats. It was therefore of particular interest to determine how endomorphins influence nociceptive processes in neuropathic pain. Therefore, we examined the antinociceptive effect of endomorphins on rats with a crush injury to the sciatic nerve.

Crush lesioning was performed in rats under pentobarbital anesthesia 7 days after i.t. implantation of cannulas. The right sciatic nerve was crushed with hemostatic forceps for 30 s, at a position 27 mm distal to the sciatic notch. The lesioning procedure was described in detail by De Koning *et al.*[15] The cold water allodynia test was also previously described in detail.[16] All animals with a crush injury to the sciatic nerve developed allodynia two days after the surgery. Morphine, administered i.t. in doses of 5 and 25 μg, had induced profound analgesia in control rats, but produced

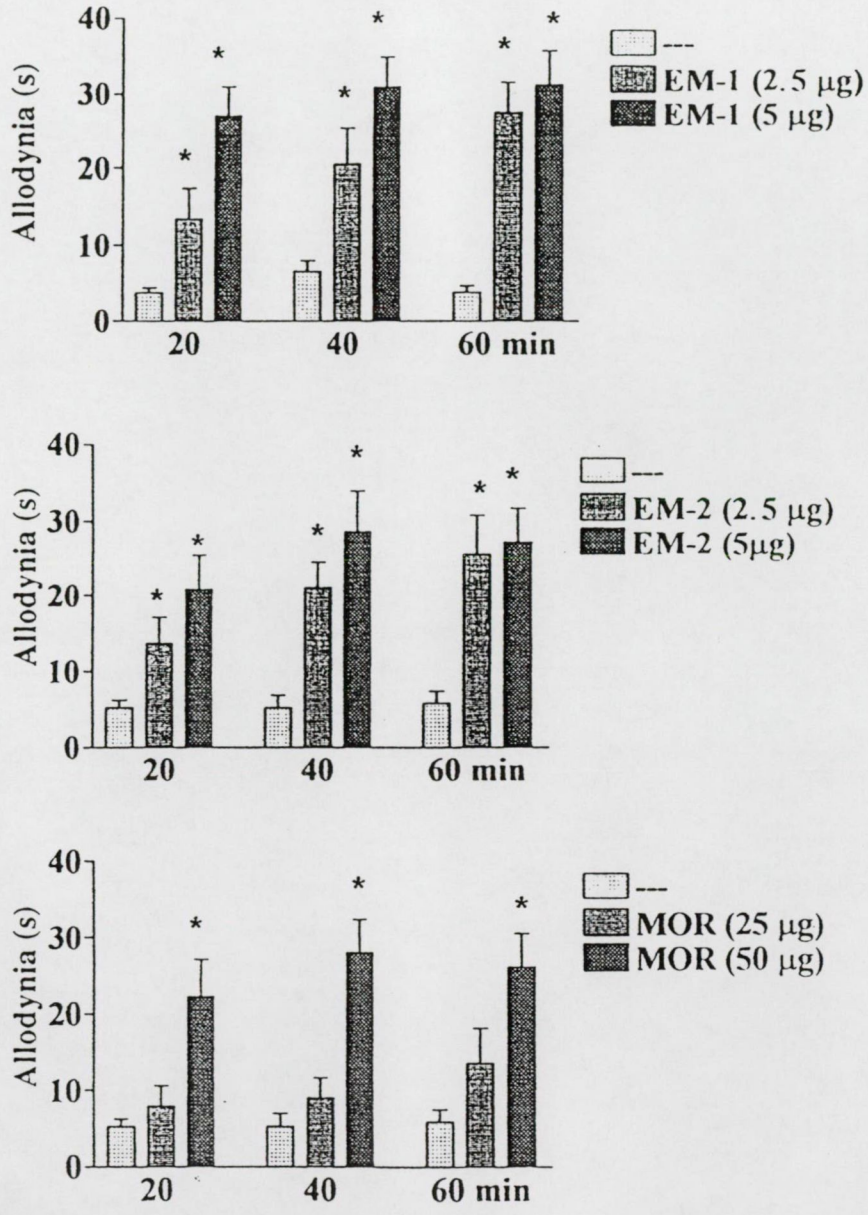

FIGURE 6. The effect of morphine (MOR), endomorphin-1 (EM-1), and endomorphin-2 (EM-2) in a cold water allodynia test in rats with a sciatic nerve injury.

no antiallodynic effect in rats with a sciatic nerve injury. Morphine produced a slight antiallodynic effect only at a dose of 50 μg (FIG. 6).

In contrast, endomorphin-1 and endomorphin-2, administered i.t. in increasing doses of 2.5 and 5 μg each, inhibited allodynia dose-dependently. The antiallodynic effect of those endomorphins was antagonized by the μ-selective antagonist cyprodime (60 μg). Thus, the present study shows that endomorphins possess anti-

nociceptive properties at a spinal cord level and display profound antiallodynia in neuropathic pain in rats.[6] Hence, the effect of endomorphins is several times stronger than that of morphine. This observation is in contrast to acute nociception, in which the potency of endomorphins is similar to that of morphine.

It is well known that the antinociceptive efficacy of morphine, administered i.t., is decreased in rats with nerve injury.[13,14,17,18] Bian *et al.*[19] confirmed that morphine, given i.t., failed to alleviate mechanical allodynia even when it was used in doses up to 100 μg in a L5/L6 ligation model of neuropathic pain. In addition, clinical studies generally indicate that neuropathic pain is somewhat resistant to morphine-induced alleviation.[20,21] It has been suggested that the ineffectiveness of morphine in models of neuropathic pain is due to the reduced number of presynaptic opioid receptors that result from the degeneration of primary afferent neurons subsequent to nerve damage.[13,14] Such reduction in the number of μ-opioid receptors may, in fact, be an important factor in diminishing the efficacy of morphine and other μ-opioid receptor agonists. Interestingly, endomorphins are particularly selective for the μ-opioid receptors and appear to be effective in neuropathic pain. The reason for such a discrepancy is still unknown because both morphine and endomorphins appear to act via the same μ-opioid receptors. Identification of the differences involved in this phenomenon may be of great importance to the understanding of the molecular mechanism of opioid action in neuropathic pain, as well as to the development of better and more effective drugs for the treatment of neuropathic pain in humans. It is possible that different μ-opioid receptor subtypes may mediate effects of morphine and endomorphins in neuropathic pain, or that molecular characterisics of μ-opioid receptors are modified by nerve injury. Furthermore, it is possible that some non-opioid receptors or mechanisms in addition to opioid receptors are involved in the analgesic effects of endomorphins in neuropathic pain.

In fact, the majority of recent studies have pointed to a great similarity in the molecular effects of morphine and endomorphins. However, the effects of endomorphins have been found to differ from those of morphine in some assays, for example, in neuropathic and to some extent in inflammatory pain.[6]

We previously showed that antinociceptive effects of various opioid receptor agonists, including morphine, were clearly enhanced by inhibition of nitric oxide (NO) syn-

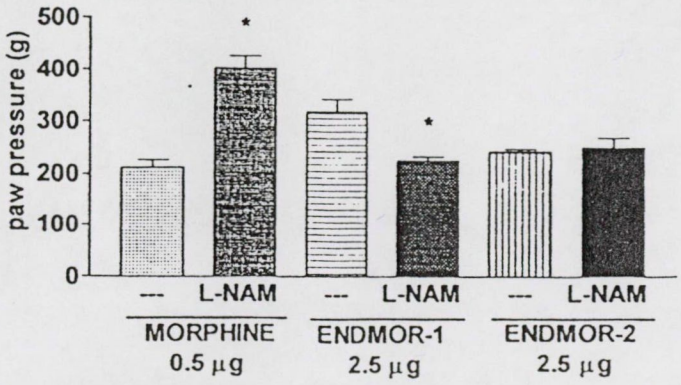

FIGURE 7. Effect of L-NAME (L-NAM, 50 μg, i.t.) on endomorphin (ENDMOR) and morphine analgesia.

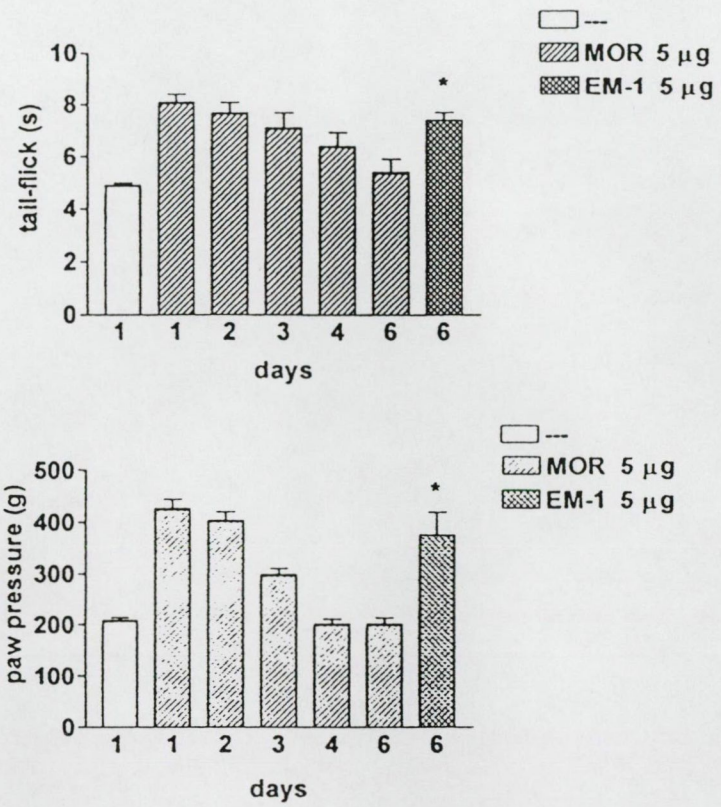

FIGURE 8. Endomorphin-1 (EM-1) analgesia in morphine (MOR)-tolerant rats.

thase.[22,23] In contrast, in our present study the antinociceptive effect of endomorphin-1 in the paw pressure test was inhibited, and endomorphin-2 antinociception was not changed, after pretreatment with NO synthase inhibitor L-NAME. (FIG. 7).

Furthermore, in our preliminary study, endomorphin-1 showed clear analgesic activity in morphine-tolerant rats. Thus, pretreatment with morphine did not result in the expression of cross-tolerance to endomorphin-1 analgesia (FIG. 8).

In conclusion, the result of the present study showed that the analgesic efficacy of endomorphins, novel endogenous opioid peptides with the highest affinity and selectivity for the μ-opioid receptors of all identified andogenous opioid peptides, is reduced in inflammation and increased in neuropathic pain. This observation is in contrast with the fact that the analgesic potency of morphine is reduced in states of neuropathic pain and increased in that of inflammation. Further, our preliminary study showed that in rats spinally administered with morphine, endomorphins displayed clear analgesic activity and therefore lacked cross-tolerance with morphine (FIG. 8). Mechanisms of this differential activity of endomorphins and morphine are unknown. However, it is interesting to note that endomorphins (but not morphine) cause internalization of the μ-opioid receptor, a mechanism which might be involved in the phenomenon.[24] Undoubtedly, further studies are necessary to better understand the physiology and pharmacology of endomorphin systems.

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