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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

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

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*).

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

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*.

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 (25×0.46 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

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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