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Endomorphin synthesis in rat brain from intracerebroventricularly injected [^{3}H]-Tyr-Pro: A possible biosynthetic route for endomorphins

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Abstract

In spite of concentrated efforts, the biosynthetic route of μ-opioid receptor agonist brain tetrapeptide endomorphins (Tyr-Pro-Trp-Phe-NH_2 and Tyr-Pro-Phe-Phe-NH_2), discovered in 1997, is still obscure. We report presently that 30 min after intracerebroventricular injection of 20 or 200 μCi [^{3}H]Tyr-Pro (49.9 Ci mmol^{-1}) the incorporated radioactivity was found in endomorphin-related tetra- and tripeptides in rat brain extracts. As detected by the combination of HPLC with radiodetection, a peak corresponding to endomorphin-2-OH could be identified in two of four extracts of "20 μCi" series. Radioactive peaks in position of Tyr, Tyr-Pro, Tyr-Pro-Phe or Tyr-Pro-Trp appeared regularly in both series and also in the "tetrapeptide cluster" constituted by endomorphins and their free carboxylic forms. In one of the four extracts in the "200 μCi" series a robust active peak in the position of endomorphin 2 could be detected. Intracerebroventricularly injected 100 nmol, but not 10 or 1000 nmol cold Tyr-Pro (devoid of opioid activity in vitro), caused a naloxone-reversible prolongation of tail-flick latency in rats, peaking between 15 and 30 min. We suggest that Tyr-Pro may serve as a biosynthetic precursor to endomorphin synthesis.

Keywords: Endomorphin de novo biosynthesis; Tyr-Pro substrate; HPLC; Radiodetection

1. Introduction

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), the potent and μ-opioid receptor selective agonist brain peptides, were discovered in 1997 [1]. Their regional distribution in brain and spinal cord, as well as at the periphery [2–7], the biodegradation pattern [8–11] and pharmacological properties (for recent reviews see Refs. [12,13]) have been extensively documented. The data on subcellular distribution and storage and neuronal release (particularly, its Ca^{2+}-dependency) are relatively scarce [7,14]. Endomorphins appear to have some distinguishing properties, both pharmacologically and biochemically. While other brain opioid peptides such as enkephalins and β-endorphin are full agonists both in GTP-γS binding stimulation [15] and in the pharmacological assays, endomorphins exhibit partial agonist properties both biochemically [15–17] and pharmacologically [18]. Furthermore, while the biosynthetic pathways for other vertebrate opioid peptides have already been clarified (it happens through a single- or multi-step cleavage from large molecular weight precursors with or without additional post-translational modifications [19]), the biosynthetic route of endomorphins is still obscure.

Based on the hypothesis that biosynthesis of an oligopeptide may take place also from its fragments through a specific enzymatic route, we decided to test first if intracerebroventricularly (icv) injected [^{3}H]Tyr-Pro could be incorporated into endomorphin-related peptides in the rat brain. Because our knowledge about the principles governing the penetration of oligopeptides through ependymal layer lining the ventricles and the neuronal plasma membrane is rather scanty [20–22], we had to allow for the probability that the access of Tyr-Pro to brain tissue is a low-efficacy process as indeed it was. However, using HPLC combined with radiodetection, incor-

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poration of label into endomorphin-related tri- and tetrapeptides could be detected in brain extracts 30 min after icv injection of 20 or 200 μCi [^{3}H]Tyr-Pro (49.9 Ci mmol^{-1}). Furthermore, icv injection of 100 nmol (but not 10, 300 or 1000 nmol) cold Tyr-Pro dipeptide (which, in itself, is devoid of opioid activity in vitro) caused a naloxone-reversible (i.e. opioid receptor-mediated) prolongation of tail-flick latency in rats.

2. Materials and methods

2.1. General

Animal care and experimental procedures were carried out according to the principles set by EC Directive 86/609/EEC. Experimental protocols were approved also by the Ethical Board controlling laboratory experiments at the Medical Faculty of Semmelweis University. Male Wistar/Wistar rats, weighing 110–150 g (analgesic measurements) or 170–220 g (brain extracts), were used. Rats were kept in groups of 5, in temperature-controlled (22±2 °C) unit with 12 h light–dark cycle (08.00–20.00–08.00). Standard laboratory chow and tap water were provided ad libitum.

2.2. Materials

2.2.1. Peptide synthesis

Amino acid derivatives and resins were obtained from Bachem AG (Budendorf, Switzerland). Coupling agents were acquired from Calbiochem-Novobiochem AG (Laufelfingen, Switzerland). Peptides were synthesized by manual solid-phase synthesis [23,24]. Peptide amides (endomorphin-1 and -2) were prepared on 4-methylbenzhydryl amine resin, and peptide acids (H-Tyr-Pro-OH, H-3′,5′-I-Tyr-Pro-OH, H-Tyr-Pro-Trp-OH, H-Tyr-Pro-Phe-OH, H-Tyr-Pro-Trp-Phe-OH, H-Tyr-Pro-Phe-Phe-OH) on choromethylated resin. The Boc-amino acid resin esters were prepared by Gisin methods [25]. *N*-hydroxybenztriazole and *N*,*N*-dicyclohexylcarbodiimide as coupling reagents were employed for peptide elongation. Peptides were cleaved from the resin using HF in the presence of anisole and dimethylsulphide for 60 min at 0 °C. Crude peptides were purified by semipreparative HPLC on Vydac 218TP1010 column (The Separations Group Inc., Hesperia, CA, USA) with a gradient system of acetonitrile and water containing 0.08% and 0.1% trifluoroacetic acid, respectively. The purities of peptides were assessed by TLC and analytical HPLC. The molecular weights of the peptides were confirmed by ESI-MS.

2.2.2. Tritium labelling

The precursor peptide (3′,5′-diiodo-Tyr-Pro) was synthesized manually by using the Merrifield solid-phase method on Merrifield-resin. We used Boc-protected amino acids (Boc-3′,5′-diiodo-Tyr and Boc-Pro). The crude peptide was purified by RP-HPLC on a Vydac 218TP1010 C_{18} (25×1 cm, 12 μm) column, using a linear gradient from 5% to 50% of the organic modifier (acetonitrile) within 30 min at a flow rate of 4 ml/min, with UV detection at 220 nm.

2.0 mg (3.76 μmol) of precursor peptide dissolved in 1 ml of dimethylformamide was tritiated with tritium gas using tritium manifold [23]. The reaction mixture contained 1.5 μl triethylamine and 12.2 mg $PdO/BaSO_4$ catalyst. 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 120 min and the unreacted tritium gas was then adsorbed onto pyrophoric uranium. The catalyst was filtered off on 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 RP-HPLC on a Vydac 218TP1010 C_{18} (25×1 cm, 12 μm) column to give a radioactive purity of >95%, checked by RP-HPLC on a Vydac 218TP54 C_{18} (25×0.46 cm, 5 μm) column using liquid scintillation detection on a Camberra Packard Radiomatic 505TR Flow Radiochromatography Detector with the Ultima-Flo M scintillation cocktail. The total activity of the product was measured by liquid scintillation counting. It was 4.44 GBq (120 mCi). The quantitative analysis of the pure, labelled peptide was performed by RP-HPLC and UV detector, using a calibration curve prepared with unlabelled Tyr-Pro. The calculated specific activity was 1.85 TBq/mmol (49.9 Ci/mmol).

Naloxone hydrochloride (NX) was a gift from DuPont Pharmaceuticals (Geneva, Switzerland). Tyr-Pro dipeptide stock solutions were prepared freshly each experimental day. The substance was dissolved in saline and the pH was adjusted to 4.5–5.0; the pH of saline vehicle control was adjusted accordingly. In the "20 μCi" series, [^{3}H]Tyr-Pro was dissolved in saline after the addition of 50 μl abs. ethanol, to give 2% (v/v) final ethanol concentration. In the other series no ethanol additive was used. Labelled dipeptide was injected in 5 μl volume in the first series, 20 μl in the second series.

2.3. Extraction procedures

For icv injections the coordinates originally described by Noble et al. [26] were used, delivered by a Hamilton syringe set into a Hamilton dispenser, calibrated for 5, 10 or 20 μl/step.

Brains, removed 15, 30 or 60 min after icv injected labelled Tyr-Pro, were powderized under liquid nitrogen, taken up with 1.0 ml abs ethanol and stored at −80 °C until extraction. An extraction procedure, devised originally for endomorphins [1], was used, except for sample boiling. In brief, the stored samples were solubilized in eight-fold amount of 0.08% (w/w) $Na_2S_2O_5$ solution then acetonitrile (ACN) was added to yield 25% (v/v) final acetonitrile concentration. They were mixed at room temperature overnight. The mixtures were centrifuged at 29,000 ×*g* for 20 min and the supernatants were extracted by solid-phase method, using 70% (v/v) ACN in the final step. The extracts were evaporated to dryness and dissolved in 2% ACN–98% TFA 0.1% (v/v) in water, and. the samples were analyzed by RP-HPLC.