Neo-kyotorphin (Thr–Ser–Lys–Tyr–Arg), a new analgesic peptide

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The amino acid sequence of a newly isolated pentapeptide, neo-kyotorphin from bovine brain was synthetically verified to be Thr–Ser–Lys–Tyr–Arg corresponding to the C-terminal portion of hemoglobin α-chain. The synthetic neo-kyotorphin showed the dose-dependent analgesia in mice which was approximately equal to that of Leu-enkephalin.

Neo-kyotorphin *Bovine brain* *Peptide synthesis* *Analgesia* *Kyotorphin* *Hemoglobin*

1. INTRODUCTION

An analgesic dipeptide, kyotorphin (Tyr–Arg), from bovine brain was isolated and identified in [1,2]. During the course of kyotorphin isolation, a peptide-like substance, which elicited contraction in the guinea pig ileum, was detected in the methanol-soluble fraction. This peptide-like substance termed KT-2 has been isolated and the amino acid sequence proposed. However, further studies suggested that KT-2 contained a peptide and trace of histamine [3]. Therefore, we synthesized the pentapeptide corresponding to the proposed amino acid sequence for chemical and pharmacological characterization, and found that the synthetic pentapeptide was identical to the natural compound and did not induce contraction in the guinea pig ileum. Here, we show that the amino acid sequence of this newly isolated peptide (neo-kyotorphin) has been synthetically verified to be Thr–Ser–Lys–Tyr–Arg, corresponding to the C-terminal portion of hemoglobin α-chain. Synthetic neo-kyotorphin exhibited an analgesic effect but no inhibitory effect on the electrically evoked contraction of guinea pig ileum. The hemoglobin C-terminal pentapeptide exhibits analgesic activity.

Abbreviations: Z, benzyloxycarbonyl; Z(OMe), *p*-methoxybenzyloxycarbonyl; TFA, trifluoroacetic acid; Tos, *p*-toluenesulphonyl

2. EXPERIMENTAL

2.1. *Synthesis of peptides*

The synthetic scheme is outlined in fig.1. Thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F-254 precoated plates (0.25 mm) in the solvent system; chloroform:methanol:water (8:3:1, by vol.), lower layer (R_f^1). High-performance thin-layer chromatography (HPTLC) on cellulose plate (10 × 20 cm, 0.1 mm thick) was carried out in the solvent system; *n*-butanol:pyridine:acetic acid:water (15:10:3:12, by vol.) (R_f^2). High-voltage paper electrophoresis (HVPE) was performed on Toyo no.51 filter paper at 50 V/cm for 90 min in a buffer (pH 3.6); pyridine:acetic acid:water (1:10:89, by vol.). To determine the relative mobility (R_m) of each spot, arginine was used as a standard. Reverse-phase

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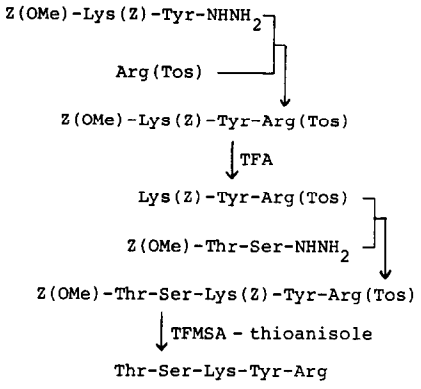

Fig.1. Synthetic scheme of neo-kyotorphin.

high-performance liquid chromatography (HPLC) was carried out using Yanaco Model L-2000 with a Cosmosil $5C_{18}$ column (15 cm × 4.6 mm) in a solvent system of 0.1 M KH_2PO_4 (pH 3.1), containing 2% acetonitrile, at 0.7 ml/min.

2.1.1. Z(OMe)-Lys(Z)–Tyr-OEt

Z(OMe)-Lys(Z) and Tyr-OEt were condensed using dicyclohexylcarbodiimide in dimethylformamide (DMF). After the coupling reaction for 48 h at room temperature, Z(OMe)-Lys(Z)–Tyr-OEt was obtained in 80% yield; m.p. 77–78°C; R_f^1 0.70; satisfactory elemental analysis for $C_{34}H_{41}O_9N_3$.

2.1.2. Z(OMe)-Lys(Z)–Tyr-$NHNH_2$

Z(OMe)-Lys(Z)–Tyr-OEt in a mixture of methanol and DMF was treated with hydrazine hydrate at room temperature overnight. Z(OMe)-Lys(Z)–Tyr-$NHNH_2$ was obtained in 84% yield; m.p. 115–118°C; R_f^1 0.68; satisfactory elemental analysis for $C_{32}H_{39}O_8N_5$.

2.1.3. Z(OMe)-Lys(Z)–Tyr–Arg(Tos)

Arg(Tos) was condensed with Z(OMe)-Lys(Z)–Tyr-$NHNH_2$ by the azide method in DMF. After the coupling reaction for 48 h at 4°C, Z(OMe)-Lys(Z)–Tyr–Arg(Tos) was obtained in 89% yield; m.p. 115–118°C; R_f^1 0.39; satisfactory elemental analysis for $C_{45}H_{55}O_{12}N_7S \cdot H_2O$.

2.1.4. Z(OMe)-Thr–Ser–Lys(Z)–Tyr–Arg(Tos)

Z(OMe)-Lys(Z)–Tyr–Arg(Tos) was deblocked with TFA–anisole, and the resulting Lys(Z)–Tyr–Arg(Tos) was condensed with Z(OMe)-Thr–Ser–$NHNH_2$ by the azide method in DMF. After the coupling reaction for 48 h at 4°C, the product was purified by silica gel column chromatography using the solvent system; chloroform–methanol–water (8:3:1, by vol., lower layer). Yield 76%; m.p. 116–119°C: R_f^1 0.22; satisfactory elemental analysis for $C_{52}H_{67}O_{16}N_9S \cdot H_2O$; satisfactory amino acid analysis of an acid hydrolysate.

2.1.5. Thr–Ser–Lys–Tyr–Arg (neo-kyotorphin)

Z(OMe)-Thr–Ser–Lys(Z)–Tyr–Arg(Tos) was deblocked with TFA–trifluoromethanesulphonic acid (TFMSA)–thioanisole–*o*-cresol at room temperature for 3 h, and the deblocked material was purified by column chromatography on Sephadex G-10 (3% acetic acid) and carboxymethyl-cellulose (a linear gradient from water to 1 M ammonium acetate). A white fluffy powder was obtained by the lyophilization in 41% yield; R_f^2 0.43; R_m 0.91; HPLC retention time 11 min; satisfactory elemental analysis for $C_{28}H_{47}O_9N_9 \cdot 2\ CH_3CO_2H \cdot 3\ H_2O$; satisfactory amino acid analyses of an acid hydrolysate and an enzymatic (leucine aminopeptidase) hydrolysate; $[\alpha]_D^{25}$ −21.7° (c = 1.2, 3% AcOH).

2.2. *Bioassay*

Analgesic effect of synthetic peptide was examined using the intracisternal injection technique in mice as in [4].

Ileal contracting activity was determined using the isolated guinea pig ileum as in [5]. Inhibitory effect on the electrically evoked contraction of the isolated guinea pig ileum was determined as in [6].

3. RESULTS AND DISCUSSION

Since diphenhydramine pretreatment inhibited the contraction of isolated guinea pig ileum induced by KT-2, we assumed that the contraction of guinea pig ileum might be induced by histamine included in the peptide sample [3]. For elucidation, we synthesized the pentapeptide corresponding to the proposed amino acid sequence by the conventional solution method. In the present synthesis, we employed the TFMSA–thioanisole deprotection system which could completely cleave the N^G-tosyl group [7]. The obtained sample was checked

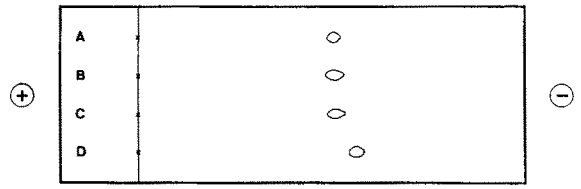

Fig.2. High-voltage paper electrophoresis: (A) natural neo-kyotorphin; (B) natural and synthetic neo-kyotorphin (A + C); (C) synthetic neo-kyotorphin; (D) arginine as a standard.

Scheme 1

137 141	hemoglobin
-----Val-Leu-Thr-Ser-Lys-Tyr-Arg	α-chain
Thr-Ser-Lys-Tyr-Arg	neo-kyotorphin
Tyr-Arg	kyotorphin
Thr-Ser-Lys-NH_2	antireproductive peptide

Amino acid sequences of neo-kyotorphin and related peptides

by amino acid analyses, elemental analysis, HPTLC, HVPE, and HPLC, and was indicated to be the desired homogeneous pentapeptide.

Synthetic pentapeptide was identical to the natural one on three different chromatographic systems; i.e., HPTLC (R_f^2 = 0.43), HVPE (R_m = 0.91) (fig.2) and HPLC (11 min). These verified that the structure of this peptide named neo-kyotorphin was Thr–Ser–Lys–Tyr–Arg.

Synthetic neo-kyotorphin exhibited no contracting effect on the isolated guinea pig ileum in a dose of 2×10^{-7} M. The electrophoretogram of KT-2 was extracted with 0.5 M acetic acid in the position in which R_m was identical to that of the authentic histamine. The extracted material induced contraction in the isolated guinea pig ileum, and diphenhydramine pretreatment inhibited this effect. Thus, it became clear that KT-2 contained both neo-kyotorphin and a trace of histamine.

Synthetic neo-kyotorphin exhibited a dose-dependent analgesic effect after intracisternal injection in mice. The ED_{50} value was 195 nmol/mouse. Analgesic activity of neo-kyotorphin is 5.6-times lower than that of kyotorphin (ED_{50} = 34.7 nmol/mouse), and approximately equal to that of Leu-enkephalin (ED_{50} = 233 nmol/mouse) [2,4,8]. However, synthetic neo-kyotorphin had no inhibitory effect on the electrically evoked contraction of the isolated guinea pig ileum in a dose of 1.3×10^{-4} M.

The peptide bond between Lys^3 and Tyr^4 in neo-kyotorphin could be cleaved by trypsin-like peptidase in the brain and Tyr–Arg (kyotorphin) was released from neo-kyotorphin [3]. Thr–Ser–Lys–NH_2 corresponding to the N-terminal portion of neo-kyotorphin (scheme 1) had been isolated from the bovine pineal glands and shown to have antigonadotropic activity [9]. Moreover, the amino acid sequence of neo-kyotorphin is identical to that of the C-terminal portion of human and bovine hemoglobin α-chain [10,11]. The hemoglobin C-terminal peptides exhibit biological activities, but the relationships between these peptides remain to be clarified.

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