Amino Acids and Peptides. XXII.[1] Preparation and Antinociceptive Effect of [D-Ala^2]Leu-Enkephalin–Poly(Ethylene Glycol) Hybrid

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The hybrid of poly(ethylene glycol) and [D-Ala^2]Leu-enkephalin was prepared by the solution method and its antinociceptive effect and inhibitory effect on the electrically induced contractions of mouse vas deferens were examined. The hybrid was synthesized by the coupling of Boc–Tyr–D-Ala–Gly–Phe–Leu–OH and amino-poly(ethylene glycol), followed by trifluoroacetic acid treatment. The hybrid showed more potent antinociceptive activity in mice and 120 times higher inhibitory activity in mouse vas deferens preparation than intact [D-Ala^2]Leu-enkephalin.

Keywords enkephalin hybrid; antinociceptive effect; poly(ethylene glycol) hybrid; mouse vas deferens

In a preceding paper, we reported that the hybrid of Leu-enkephalin (LEnk) and amino-poly(ethylene glycol) (aPEG) showed more potent analgesic activity than intact LEnk.[2] To obtain more potent analgesic, the hybrid of [D-Ala^2]Leu-enkephalin ([DAla^2]LEnk) and aPEG was prepared, as shown in Fig. 1, based on our finding that the analgesic effect of [DAla^2]LEnk was more potent than that of LEnk.[3] aPEG was prepared from PEG #4000 (MW, 3000—3700) according to the procedure reported by Pillai and Mutter[4] and was purified by Dowex 50 (H^+ form) column chromatography as described.[2] The amino content of aPEG ranged among lots from 0.26 to 0.56 meq/g. The reason why PEG #4000 was selected from among many kinds of poly(ethylene glycol) (PEG) was based on our speculation that the bulky PEG portion was not huge enough to hinder the binding of the peptide portion to its receptor. β-Endorphin, a potent analgesic containing the enkephalin (Enk) sequence, has a molecular weight of 3294, which does not greatly differ from that of the LEnk–aPEG (#4000) hybrid. Boc–Tyr(Bzl)–DAla–Gly–Phe–Leu–OBzl was prepared by stepwise elongation from Boc–Gly–Phe–Leu–OBzl[5] using the mixed anhydride method.[6] The protected pentapeptide was hydrogenated to give Boc–Tyr–DAla–Gly–Phe–Leu–OH, coupled with aPEG by the dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) method.[7] The product was purified by LH-20 column chromatography, followed by trifluoroacetic acid (TFA) treatment to remove the Boc group. The final product was purified by LH-20 column chromatography using a mixture of methanol–dichloromethane (MeOH–DCM) as an eluent, followed by reverse-phase, high-performance liquid chromatography (RP-HPLC). The peptide content of the hybrid was 0.39 mmol/g.

Inhibitory effects of the hybrid and [DAla^2]LEnk on electrically induced contraction of the mouse vas deferens (MVD) are shown in Table I.

IC_{50} of the hybrid was calculated as the concentration of [DAla^2]LEnk contained in the hybrid. As shown in the Table, the inhibitory effect of the [DAla^2]LEnk on MVD contraction was enhanced 120 times by hybrid formation with aPEG. Of LEnk, 12.0 nmol equals 7.1 μg; of [DAla^2]LEnk, 6.08 nmol is 3.7 μg; and of the hybrid, 0.05 nmol is 0.13 μg. It can thus be said that the effect of the hybrid was more potent than that of [DAla^2]LEnk even in terms of weight ratio.

The antinociceptive effect of the hybrid administered intracerebroventricularly (i.c.v.) was examined by the tail-pinch method as illustrated in Figs. 2 and 3. Neither PEG nor aPEG had any appreciable antinociceptive effect in the test. [DAla^2]LEnk produced no prominent effect at doses up to 30 nmol/animal: however, the hybrid at a dose of 10 nmol/animal exhibited a remarkable anti-

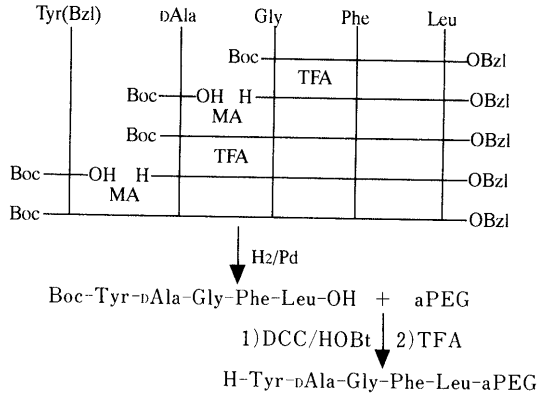

Fig. 1. Synthetic Scheme for the Hybrid of [DAla^2]LEnk and aPEG
MA: mixed anhydride method.

TABLE I. Inhibitory Effects of the Hybrid on the Electrically Induced Contractions of MVD

	MVD	
	IC_{50} (nM)	Relative activities
LEnk	12.0	100
[DAla^2]LEnk	6.08	197
[DAla^2]LEnk–aPEG	0.05[a)]	24000

a) Calculated as [DAla^2]LEnk contained in the hybrid.

nociceptive effect. The effect of 10 nmol of the hybrid was equipotent to that of 100 nmol of [DAla^2]LEnk. So it can be said that the antinociceptive effect of the [DAla^2]LEnk was enhanced by hybrid formation with aPEG 10 times in terms of molar ratio.

As shown in Fig. 3, the hybrid showed prolonged effect and lasted at least 1 h.

The enhancement of antinociception by hybrid formation may be based on slower enzymatic degradation and more stable receptor-binding of the hybrid as compared with intact [DAla^2]LEnk, although its mechanism is yet to be fully understood.

PEG is an attractive candidate for a drug carrier since it is stable, bioinert and only weakly immunogenic. The hybrid of a small peptide with a PEG is expected to be an effective and long-acting drug.

MATERIALS AND METHODS

Melting points are uncorrected. Solvent systems for ascending thin-layer chromatography on Silica gel G (type 60, Merck) are indicated as follows: Rf^1 = BuOH–AcOH–H_2O (4:1:5, upper phase), Rf^2 = BuOH–pyridine–AcOH–H_2O (4:1:1:2), Rf^3 = $CHCl_3$–MeOH–H_2O (8:3:1, lower phase), Rf^5 = $CHCl_3$–AcOH–MeOH (90:2:8). Synthetic peptide was hydrolyzed in 6 N HCl at 110 °C for 20 h and the hybrid was hydrolyzed for 48 h. Amino acid compositions of acid hydrolysates were determined with a Hitachi 835 amino acid analyzer. Rotations were measured with a JASCO DIP-360 polarimeter. RP-HPLC was conducted with a Waters 600 on YMC Pack AQ-ODS-5 using a mixture of 0.1% TFA-containing CH_3CN/H_2O as an eluent. PEG #4000 was purchased from Nacalai Tesque Inc. and was converted to aPEG according to a procedure reported by Pillai and Mutter.[4] aPEG was purified as reported in the preceding paper.[2]

Boc–DAla–Gly–Phe–Leu–OBzl Boc–Gly–Phe–Leu–OBzl[8] (4 g, 8 mmol) and anisole (0.4 ml) were dissolved in TFA (4 ml) and the solution was stirred for 1 h at 0 °C. A mixture of ether and petroleum ether (1/1) were added and the resulting precipitate was dried *in vacuo*. The material was dissolved in DMF (10 ml) and was neutralized with Et_3N (1.1 ml, 8 mmol).

Isobutylchloroformate (1.0 ml, 8 mmol) was added to a solution of Boc–DAla–OH (1.51 g, 8 mmol)and Et_3N (1.1 ml, 8 mmol) at −10 °C and the mixture was stirred for 15 min. The reaction mixture was added to the tripeptide solution and the whole was stirred for 16 h. The solvent was removed *in vacuo* and the residue was extracted with AcOEt, followed by washing with 5% citric acid, 5% Na_2CO_3 and H_2O. After drying with Na_2SO_4, the solvent was removed *in vacuo* and the residue was precipitated from AcOEt–petroleum ether. Yield: 4 g (88%), mp 109—113 °C, $[\alpha]_D^{24}$ −21.4° (c=1.0, MeOH). *Anal.* Calcd for $C_{32}H_{44}N_4O_7$: C, 64.39; H, 7.45; N, 9.39. Found: C, 64.70; H,7.59; N,9.41. Amino acid ratios in an acid hydrolysate: Ala 0.96, Gly 1.02, Phe 1.00, Leu 1.09 (average recovery 94%).

Boc–Tyr(Bzl)–DAla–Gly–Phe–Leu–OBzl Boc–DAla–Gly–Phe–Leu–OBzl (3 g, 5 mmol) was deblocked with TFA. The deblocked material was neutralized with Et_3N in DMF and reacted with the mixed anhydride prepared from Boc–Tyr(Bzl)–OH (2.8 g, 7.5 mmol) in the usual manner. After 2 d, the solvent was removed and the residue was extracted with AcOEt, followed by washing with 5%

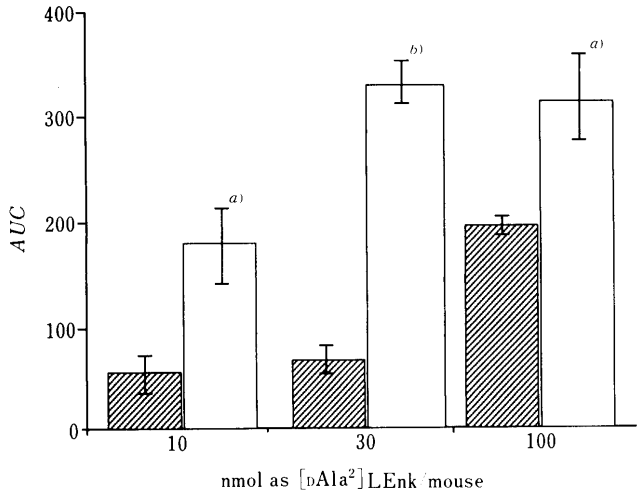

Fig. 2. Antinociceptive Effect of i.c.v. Administered [DAla^2]LEnk–aPEG Examined by the Tail Pinch Method

▨, [DAla^2]LEnk; □, [DAla^2]LEnk–aPEG.
a) $p<0.05$, *b*) $p<0.01$, compared with the matched [DAla^2]LEnk group.

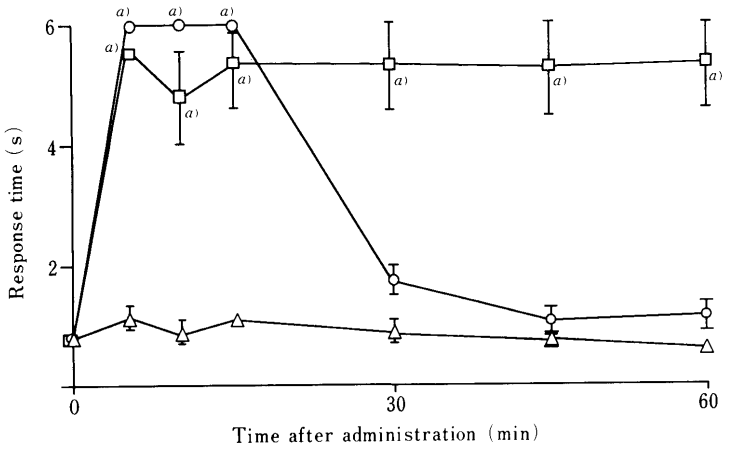

Fig. 3. Antinociceptive Effects of i.c.v. Administered Peptides and Hybrid Examined by the Tail Pinch Method

□, [DAla^2]LEnk–aPEG (100 nmol as peptide/mouse); ○, [DAla^2]LEnk (100 nmol/mouse); △, LEnk (100 nmol/mouse).
a) $p<0.01$, compared with the respective pretest value.

citric acid, 5% Na_2CO_3, and H_2O. After drying over Na_2CO_3, the AcOEt was removed and the residue was precipitated from AcOEt–petroleum ether. Yield : 2.8 g (68%), mp 142—147 °C, Rf^3 0.83, $[\alpha]_D^{24}$ −39.8° (c=1.0, DMF), *Anal.* Calcd for $C_{48}H_{59}N_5O_9 \cdot 2H_2O$: C, 65.05; H, 7.18; N, 7.90. Found: C, 65.20; H, 6.99; N, 8.14. Amino acid ratios in an acid hydrolysate: Tyr 0.77, Ala 1.00, Gly 1.02, Phe 1.00, Leu 1.09 (average recovery 88%).

Boc–Tyr–DAla–Gly–Phe–Leu–OH Prepared from Boc–Tyr(Bzl)–DAla–Gly–Phe–Leu–OBzl (1.5 g, 1.76 mmol) by hydrogenation with Pd in MeOH in the usual manner. Yield: 1.07 g (91%), mp 128 °C, Rf^3 0.44, $[\alpha]_D^{24}$ 10.4° (c=1.0, DMF). *Anal.* Calcd for $C_{34}H_{47}N_5O_9$: C, 60.96; H, 7.09; N, 10.46. Found: C, 60.67; H, 6.89; N, 10.49. Amino acid ratios in an acid hydrolysate: Tyr 0.84, Ala 1.10, Gly 1.07, Phe 1.00, Leu 1.04 (average recovery 84%).

H–Tyr–DAla–Gly–Phe–Leu–aPEG DCC (70 mg,0.34 mmol) was added to a solution of Boc–Tyr–DAla–Gly–Phe–Leu–OH (188 mg, 0.28 mmol), HOBt (46 mg, 0.34 mmol), and aPEG (amino content: 0.28 meq/g) (500 mg, amino content 0.14 meq) in DMF–DCM (1 : 1, 8 ml) and the mixture was stirred. After 12 h, additional DMF solution (4 ml) of Boc–Tyr–DAla–Gly–Phe–Leu–OH (282 mg, 0.42 mmol), HOBt (69 mg,0.51 mmol) and DCC (105 mg, 0.51 mmol) was added and the whole was stirred for additional 2 d. The solvent was removed and the residue was dissolved in a mixture of DMF and DCM (1 : 1, 5 ml). Insoluble material was filtered off and the solution was passed through Sephadex LH-20 column (3.2 × 132 cm). The solvent was removed and the residue was lyophilized from dioxane to give 600 mg of fluffy powder. Rf^3 0.63. The powder (580 mg) was treated with TFA (1 ml) containing anisole (0.05 ml) and *m*-cresol (0.05 ml) for 1 h at 0 °C. The TFA was removed *in vacuo* and the residue was dissolved in 1% AcOH. The solution was passed through Sephadex LH-20 column (3.2 × 140 cm). Five ml fractions were collected and absorbance of each fraction at 280 nm was checked. Fractions 97—115 were pooled and the solvent was evaporated off. The residue was lyophilized from HCl-containing H_2O. Yield : 548 mg. The material was further purified by RP-HPLC on a YMC Pack AQ-ODS-5 using a mixture of 0.1% TFA-containing CH_3CN/H_2O as an eluent. Yield : 247 mg, fluffy powder, Rf^3 0.45. Amino acid ratios in an acid hydrolysate: Tyr 0.93, Ala 0.96, Gly 1.14, Phe 1.00, Leu 1.07. The peptide content: 0.39 mmol/g.

Bioassay [DAla^2]LEnk, [DAla^2]LEnk–aPEG hybrid and morphine hydrochloride (Takeda) were dissolved in saline so that the dose was contained in a volume of 0.1 ml/10 g of body weight. LEnk, the hybrid and morphine were administered i.c.v., according to the method of Haley and McCormick.[8]

Male mice of ddY strain weighing 18 to 20 g (Ohtsubo Experimental Animals) were purchased and housed in a temperature-controlled room with free access to food and water. After reaching 23—28 g and 30—35 g, they were used for *in vivo* and *in vitro* experiments, respectively.

In *in vitro* experiments, MVD was rapidly isolated from mice weighing 30—35 g and mounted in a 10 ml organ bath filled with Mg^{2+}-free Krebs Henseleit solution kept at 37 °C.[9] The inhibitory effect of the test compounds on the electrically induced contractions of the preparation were estimated from a concentration-response curve and expressed as IC_{50}. Details of the procedure were reported in our previous paper.[10]

The antinociceptive effect was measured by a modification of Haffner's method,[11] using a cut-off time of 6 s to avoid the tissue damage. The measurement was made at intervals of 15 min for 60 min after administration of test compounds. The effect was calculated as the area under the curve (*AUC*) by plotting the response (S) on the ordinate and elapsed time (min) on the abcissa.

Statistical significance of the differences was evaluated by Student's t-test or by the analysis of variance followed by Dunnett's test for individual comparisons.

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REFERENCES AND NOTES

1) Standard abbreviations for amino acids, peptides and protecting groups are used [*Eur. J. Biochem.*, **138**, 9 (1984)]; other abbreviations include: DMF=dimethylformamide, Et_3N=triethylamine, DAla=D-Ala.
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