Solid Phase Synthesis and Opioid Receptor Binding Properties of Presumable Dermorphin Precursor Derivatives

Yusuke SASAKI, Akihiro AMBO and Kenji SUZUKI*

Tohoku College of Pharmacy, 4-1, Komatsushima 4-chome, Aoba-ku, Sendai 981, Japan. Received October 24, 1990

Presumable dermorphin precursor peptide derivatives comprised of 35 amino acids and their fragments, which are based on the amino acid sequence determined by recombinant deoxyribonucleic acid (DNA) techniques, were synthesized by the solid phase method. A 35-residue peptide amide containing L-Ala¹-dermorphin sequence at the N-terminus (1) as well as its D-Ala¹ isomer (2) and the C-terminal 20-residue peptide amide were found to be unexpectedly stable against aminopeptidase M digestion and in rat brain membrane fractions mixture, suggesting that the C-terminal Glu-rich moiety of 1 and 2 serves to protect from enzymatic breakdown. In the opioid receptor binding assay, 2 showed 40 and 25-fold higher affinities than 1 for µ and δ-receptors, respectively. The N-terminal 15-residue peptide fragment of 2 showed greatly increased affinities for both receptors, being one half of those of dermorphin, whereas that of 1 showed low affinities. Opioid receptor binding properties of these synthetic peptides may be useful in investigation of the processing to dermorphin.

**Keywords** dermorphin precursor peptide; 35-residue peptide amide; 15-residue peptide; solid phase synthesis; receptor binding assay; enzymatic stability

Dermorphin (Tyr–D-Ala–Phe–Gly–Tyr–Pro–Ser–NH₃) is a highly potent opioid peptide isolated from the South American frog.² By using a complementary deoxyribonucleic acid (cDNA) library prepared from the skin of Phyllomedusa sauvagaei, the amino acid sequence of several dermorphin precursor polypeptides were recently determined.³ Among these precursors, a polypeptide deduced from cDNA termed D-1/2 comprised 197 amino acid residues. As shown in Fig. 1, in the C-terminal fragment of this prepro-dermorphin, four copies containing dermorphin sequence are present. Three copies of these are 33-residue peptide flanked by the typical pro-hormone processing signal (Lys–Arg) at the amino and carboxyl ends and contain additional processing signals (Lys–Lys and Lys–Arg) in the polypeptide chain. A processing cascade including epimerization of Ala residue to D-Ala from the prepro-dermorphin to dermorphin is yet unknown.

The present paper deals with the synthesis and opioid receptor binding properties of presumable processing intermediate peptides or peptide derivatives. The peptides synthesized are shown in Fig. 2. The 35-residue peptide (1 and 2) and their C-terminal 20-residue peptide (5) were synthesized as the C-terminal amide form to enhance enzymatic stability during biological assays. In general, it is also well known that C-terminal amide derivatives of opioid peptide do not decrease the biological activity dramatically.

Peptides were synthesized by the solid phase method. Boc-amino acids with following side chain protecting groups were used: Tos for Arg, Cl–Z for Lys, cHx for Glu, Bzl for Ser, Br–Z for Tyr and DNP for His. For the synthesis of 1, 2 and 5, starting with benzhydrilamine resin, double coupling was performed at each residue with 4-fold excess symmetrical anhydride except for the incorporation of Boc–Asn which was effected with the HOBr/DIPCDI⁵ method. The peptide was cleaved from the resin, with simultaneous side chain deprotection, by treatment with a mixture of HF/p-cresol/p-thiocresol followed by treatments with dil. alkaline solution to reverse any N→O shift and with 20% β-mercaptoethanol to remove the DNP group from the His residue.⁶ Purification of peptides was performed first by gel filtration on Sephadex G-25 and then by preparative medium-pressure HPLC on Develosil LOP ODS. Analytical HPLC of crude 35-peptides (1 and 2) gave similar elution patterns. The HPLC profiles of crude and purified 2 are shown in Fig. 3. Peptides 3 and 4 were analogously synthesized starting with Boc–Arg(Tos)– Merrifield resin with some modifications that the peptide assembly was performed by single coupling after the incorporation of Lys¹¹ residue and the peptide was cleaved from the resin with a mixture of HF/anisole. Amino acid ratios of all synthetic peptides after acid hydrolysis agreed well with those of expected values. Interestingly, 1, 3 and 5, which did not contain D-amino acid in the mole-

---

**Fig. 1. Schematic Representation of a Dermorphin Precursor Polypeptide (D-1/2)**

B1, B2 and B3 are highly homologous: only one or two residues of B3 region are substituted at respective positions of B1 (1, E¹⁵⁵–V) and B2 (*, N¹⁷⁰–K and N¹⁷²–I) regions. A region is preceded by dermorphin (or dermenkephalin, ref. 4) sequence (YMPHLLMD). , signal peptide; , dermorphin sequence; , dermorphin sequence.

Chou–Fasman’s secondary structure prediction: , random coil; , β-turn; , α-helix.

© 1991 Pharmaceutical Society of Japan
Opioid receptor binding affinities of peptides were determined by competition experiments with rat brain membrane fractions using radioligands, $[^3H]$DAGO (μ) and $[^3H]$DADLE (δ), and the results are shown in Table II. 1 showed very weak affinities for μ and δ-receptors. 5 showed virtually no affinities for both receptors. On the
other hand, 2 showed 40 and 25-fold higher affinities than those of 1 for \( \mu \) and \( \delta \)-receptor, respectively. Further, its N-terminal fragment peptide 4, showed greatly increased \( \mu \) and \( \delta \)-receptor affinities, being 500-fold those of 1 and 3, while 1 did not changes affinities by lacking its C-terminal 20-residue peptide amide. It seems unlikely that the enzymatic breakdown of 1 and 3 during binding assay is responsible for the low affinity because the hydrolysis rates under conditions similar to the binding assay\(^9\) are 1: <5% and 3: <20% when assessed by HPLC. The \( K(D)/K(I) \) values of synthetic peptides (1–4) were 1–4, which indicate \( \mu \)-selectivity of these peptides though their \( \mu \)-affinities and \( \mu \)-selectivities were considerably lower than that of dermorphin. It should be noted that 3 had low but significant affinities for \( \mu \) and \( \delta \)-receptors whereas \( \lambda \)-Ala\(^2\)-dermorphin has been found to be devoid of biological activities\(^1\) and rat brain membrane binding affinity.\(^1\)

In conclusion, the present study demonstrate that a presumable dermorphin precursor 15-residue peptide extended at the C-terminus of dermorphin (4) had high affinities, being one half of those of dermorphin, for \( \mu \) and \( \delta \)-receptors in rat brain. Several experimental data have been accumulated to date showing the presence of immunoreactive dermorphin or its higher molecular forms in mammalian tissues.\(^1\) It is of interest to know the step of the processing cascade in which the epimerization of Ala\(^2\) residue of dermorphin occurs. In this respect, the synthetic peptides reported here may be useful in investigation of the processing to dermorphin.

**Experimental**

Optical rotations were measured by a JASCO DIP-40 polarimeter. Amino acid analysis was performed using a Hitachi 835 amino acid analyzer after 6N HCl hydrolysis of the peptide in an evacuated sealed tube at 110 °C for 25 h. Analytical HPLC were performed on a YMC ODS column (AM-303-100, 25 x 4.6 mm) by gradient elution using the following solvent systems: A: 0.5% TFA and B: 80% acetonitrile containing 0.06% TFA. A linear gradient from 10% B to 45% B over 40 min at a flow rate of 1.2 mL/min was used and the eluate was monitored at 215 nm.

**Solid Phase Synthesis**

Solid phase synthesis was carried out on benzhydromine resin hydrochloride (0.6 mmol/g, 1% cross link) or Boc-Arg(Tos)-Merrifield resin (0.2 mmol/g, 1% cross link) using a Biosearch 9600 peptide synthesizer according to a coupling schedule as shown in Table III. For the double coupling, steps 10—14 in Table III were repeated before the capping step (15).

**Isolation of Peptides**

Peptides 1, 2 and 5 were obtained in essentially the same way. For example, protected \( \lambda \)-Ala\(^2\)-containing peptide resin (600 mg) was treated with a mixture of HF/p-cresol/p-thiocresol (90 : 5 : 5, 15 ml) at -20 °C for 30 min and then at 0 °C for 60 min. After evaporation of HF under reduced pressure the resulting residue was triturated with abs. ether and extracted with 10% AcOH. The extract was evaporated to dryness. The product was treated with diisopropylamine (DIP) at 0 °C for 60 min and then with 20% aqueous \( \beta \)-mercaptoethanol at room temperature for 2 h. The entire solution was concentrated to small volumes, when some insoluble materials were removed by centrifugation, and the resulting solution was applied to a column (2.5 x 90 cm) of Sephadex G-25. The column was eluted with 10% AcOH and Pauly reaction-positive eluent was collected and lyophilized to yield a colorless fluffy material, 290 mg. The product (150 mg) was dissolved in 1% TFA (1 ml), when some insoluble materials were removed by centrifugation, and applied to a column (3 x 30 cm) of Sephrose LOP ODS which was eluted with a linear gradient from 12% to 28% acetonitrile in 0.06% TFA over 150 min at a flow rate of 3 ml/min. Fractions of 6 ml each were collected and the Nos. 55—57 were pooled, evaporated to dryness and lyophilized from H2O to a yield of 2 as a colorless fluffy material, 16.5 mg (7.8% based on the starting resin), \([\alpha]_D^{25} = 50.2^\circ \) (c=0.32, 1% AcOH), HPLC-t\(_g\) 25.4 min.

**References and Notes**

1. Amino acids and peptides are of \( L \)-configuration unless otherwise noted. Abbreviations used are: Boc= tert-butylxycarbonyl, Tos= tosyl, Cl=Z=2-chlorobenzylxycarbonyl, Cbz= cyclohexyl, Bzl= benzyl, Br-Z=2-bromobenzylxycarbonyl, DNP= dimethylpyridinium, HOBt=1-hydroxybenzotriazole, DIPCDI= N,N’-disopropylcarbodiimide, DIPA= N,N’-disopropropylylenethyamine, TFA= trifluoroacetic acid, DCM= dichloromethane, DMF= dimethylformamide, DAGO=\( [\lambda]-\)Ala\(^2\), MePhe\(^2\), Gly\(^2\)-[\gamma]-benzylpenicillin, DACLE=\( [\lambda]-\)Ala\(^2\), \( \beta \)-Leu\(^2\), penicillin, HPLC= high performance liquid chromatography, HPLC-t\(_g\)= retention time on analytical HPLC, BSA= bovine serum albumin.