Enkephalin Alkylamides As Dimeric Probes for Bivalent Interaction with δ Opioid Receptors[1)]

Yasuyuki SHIMOHIGASHI,* Ayako TANI, Motonori OHNO, and Tommaso COSTA†
Laboratory of Biochemistry, Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka 812
† Laboratory of Pharmacology, Sanita National Institute, Rome, Italy.

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The bifunctional interaction of dimeric enkephalins with δ opioid receptors was evidenced by comparing activities between dimers and their monomeric alkylamide analogs having alkyl groups corresponding to the crosslinking spacer. When the tetrapeptide enkephalin amide H–Tyr–D-Ala–Gly–Phe–NH_2 was alkylated by a dodecyl group, the resulting enkephalin dodecylamide exhibited only 7—15% activities of those of the nonalkylated amide in the μ assays (muscle assay in guinea pig ileum and rat brain binding assays using [^{3}H]naloxone or [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin) and 6—48% activities in the δ assays (muscle assay in mouse vas deferens and binding assays using [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin or [^{3}H]-[D-Ser^2,Leu^5]enkephalyl-Thr^6). DTE_{12}, a dimer of the structure (H–Tyr–D-Ala–Gly–Phe–NH–$(CH_2)_6$–)$_2$ which is created structurally by linking the second tetrapeptide enkephalin molecule to the dodecyl group of enkephalin dodecylamide, was almost as active as enkephalin dodecylamide in the μ assays. However, its δ activities were drastically enhanced, showing 57-fold increase in activity in the muscle assay and 33-fold increase in the brain binding assays when compared with the dodecylamide monomer. Similar results were also obtained for a set of pentapeptide enkephalins; i.e., enkephalin amide H–Tyr–D-Ala–Gly–Phe–Leu–NH_2, its ethylamide analog and crosslinked dimer DPE_2. The results indicated that the presence of the second peptide in the dimeric enkephalin molecule is responsible for most of increase in biological activities and receptor selection, and that the dimeric enkephalin functions as bivalent ligands for δ receptors.

A bivalent ligand with two biologically active cores that are crosslinked by a spacer would be capable to interact with two receptors simultaneously. This might result in an increase in affinity, specificity, selectivity, and probably biological activity. The studies using bivalent ligands have revealed that the receptor crosslinking or microaggregation may be important to elicit some intrinsic biological responses.[2)] We have indeed reported that the double headed enkephalins in which two enkephalin molecules are crosslinked at the COOH terminus by α,ω-diaminoalkanes displayed a greater affinity and selectivity for the δ or μ opioid receptor binding sites.[3—8)] The receptor selection of dimers depended on the sequences for dimerization and on the length of crosslinking spacer. In the radio-ligand binding assays, a dimeric pentapeptide enkephalin DPE_2[9)] (Fig. 1) exhibited several fold increase in affinity with δ sites when compared with monomeric analogs, whereas its μ activities were almost unchanged.[3)] When μ-selective desLeu^5-tetrapeptide enkephalin amide was dimerized, the dimer DTE_{12} showed a greater selectivity for δ sites.[4)] The enhanced affinity and selectivity of DPE_2 and DTE_{12} have suggested that two δ receptor binding sites are in a close proximity. Similar results were obtained also for μ receptors with dimers of tripeptide enkephalins.[10)]

The bivalency of these dimeric enkephalins must be proved regarding their subsite interactions, kinetics, thermodynamical potentials, and more directly the bifunctional interactions with receptors. Since the crosslinking spacer moiety *per se* may also interact with receptors or biomembranes, possible bifunctional receptor interaction of dimeric enkephalins should be confirmed by examining the contributions of the spacer and the second enkephalin molecule to receptor responses, respectively. This requires to inspect the activities of analogs having only the spacer molecule without the second peptide moiety. In this study, in order to elucidate the bivalency of enkephalin dimers for δ receptors, we have designed and synthesized a tetrapeptide enkephalin dodecylamide TE_{12} and a pentapeptide enkephalin ethylamide PE_2 (Fig. 1).

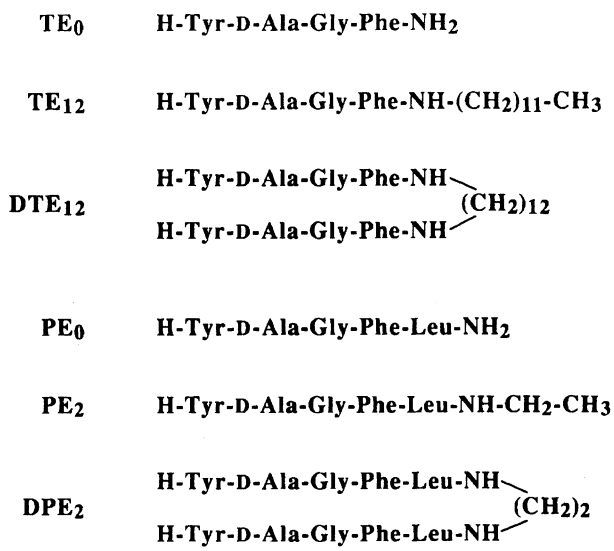

Fig. 1. Structure of monomeric and dimeric enkephalin analogs.

The activities were evaluated and compared in the in vitro muscle assays using the electrically stimulated guinea pig ileum (GPI) and mouse vas deferens (MVD),

and in the radio-ligand binding assays using rat brain membrane preparations. The results clearly indicated that the attachment of the second enkephalin molecule to enkephalin alkylamide increases dramatically the affinity only for δ receptors.

Results and Discussion

The synthetic scheme of TE_{12} is shown in Fig. 2. Dodecylamine was coupled with Boc–Phe–OH by the carbodiimide method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride and 1-hydroxybenzotriazole (HOBt). Boc–Phe–Leu–ethylamide was prepared in a similar way. After removal of the Boc group, these alkylamides were coupled with tripeptide acid Boc–Tyr–D-Ala–Gly–OH also by the EDC–HOBt method. The resulting enkephalin alkylamides were liberated by treatment with trifluoroacetic acid (TFA) and purified by gel filtration. Purity was verified by high-performance TLC, HPLC, and amino acid analysis.

Table 1 shows the potencies of TE_{12} and DTE_{12} relative to their nonalkylated monomeric analog TE_0. The results are appreciably consistent between muscle and brain binding assay systems. When the amide group of TE_0 was alkylated by a dodecyl group, the resulting TE_{12} lost dramatically the affinity for both δ and μ receptors. When compared with TE_0, the δ activities of TE_{12} were only 6% in MVD, 48% in [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (^{3}H-DADLE) and 29% in [^{3}H]-[D-Ser^2,Leu^5]enkephalyl-Thr^6 (^{3}H-DSLET) binding assays, while its μ activities were 7% in GPI, 8% in [^{3}H]naloxone (^{3}H-NAL) and 15% [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]-enkephalin (^{3}H-DAGO) binding assays. ^{3}H-DSLET is a highly δ-specific ligand with low nonspecific bindings. Although DADLE is less δ-selective than DSLET, the binding affinities of TE_0 and TE_{12} are almost comparable both in ^{3}H-DADLE and ^{3}H-DSLET binding assays.

The dimer DTE_{12} is structurally created by attachment of another tetrapeptide enkephalin molecule toward TE_{12}. DTE_{12} exhibited μ activities (6—17%) which are comparable to those of TE_{12}. However, there were remarkable increases in δ activities in both muscle and binding assays (Table 1). DTE_{12} was 57 times more potent than TE_{12} in the MVD assay, and 33 times more potent in the ^{3}H-DADLE and ^{3}H-DSLET binding assays. These results clearly demonstrate that an attachment of the second molecule of enkephalin is responsible for a quite favorable interaction only with δ receptors, but not with μ receptors. In other words, the increase in affinity of DTE_{12} to δ receptors requires the simultaneous presence of two enkephalin molecules that are crosslinked by a dodecamethylene group.

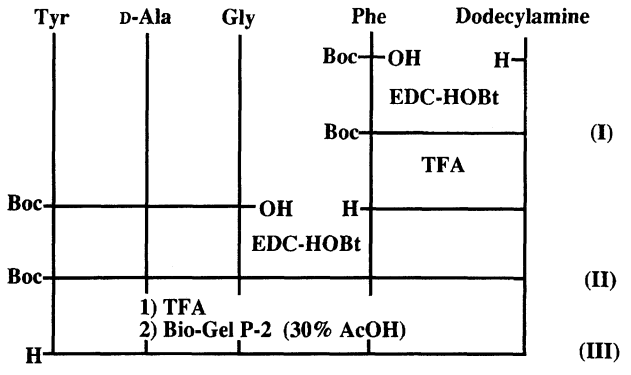

Fig. 2. Synthetic scheme of tetrapeptide enkephalin dodecylamide TE_{12}.

Similar results were also obtained from a pentapeptide enkephalin series (Table 2). DPE_2 was about 20 times more active than PE_2 in the MVD assay and about 5 times more active in the ^{3}H-DADLE and ^{3}H-DSLET binding assays. Its μ activities varied in different assays, but the potency differences did not exceed a 100% (Table 2).

The contribution of alkyl groups in the receptor affinity seems to be different between TE_{12} and PE_2. This might be caused by the difference in the number of methylenes in alkyl groups. Increase in hydrophobicity or lipophilicity would increase the nonspecific adsorption of compound to tissue membranes. It should be noted that TE_{12} retains significant activities (48% and 29% of TE_0) in the ^{3}H-DADLE and ^{3}H-DSLET assays, respectively, while its potency in the MVD assay diminished to 6% of TE_0. PE_2 did not show such a reduction in activity. These results imply that there might be nonspecific interactions between dodecyl group and muscle tissues, and thus the activity in the muscle assays may not necessarily reveal real receptor activity.

Similar phenomena are also observed for DTE_{12}. The wash-out process of peptides in the muscle assay was extremely difficult for DTE_{12}, suggesting the presence of nonspecific interactions.[1] DPE_2 did not show such a difficulty in wash-out. When the δ-activities of dimers were compared with those of their nonalkylated amides in the binding assay, DTE_{12} was 10—16 times more active than TE_0 and DPE_2 was 2—6 times more potent than PE_0. In the MVD assay, the increase was only 3 times for DTE_{12}, whereas DPE_2 displayed an even more increased activity (30-fold). Such unmatched enhancements in δ-activity between DTE_{12} and DPE_2 is due probably to their difference in the size of methylene bridges, which are directly related to the molecular hydrophobicity.

In spite of hydrophobic interaction of dimers at their spacer moiety, the effect of peptide dimerization on increase in specificity and selectivity for δ receptors is quite apparent for both tetrapeptide and pentapeptide enkephalins. Dimeric enkephalins function as genuine bivalent ligands for δ receptors. Several lines of examination have shown the biochemical and biophysical behaviors of dimers different from those of monomers.[5—8] For instance, using a radio-labeled dimer ^{3}H-DPE_2, it was found that the dissociation rate of ^{3}H-DPE_2 in NG 108-15 cells was about twice smaller than the monomer

Table 1. Opioid Activities of Monomeric and Dimeric Tetrapeptide Enkephalin Analogs in Muscle Assays and Rat Brain Binding Assays

Peptide	Relative μ-activity			Relative δ-activity		
	GPI	^{3}H-NAL	^{3}H-DAGO	MVD	^{3}H-DADLE	^{3}H-DSLET
TE_0	100	100	100	100	100	100
	(90)	(1.1)	(1.5)	(130)	(39)	(43)
TE_{12}	7	8	15	6	48	29
DTE_{12}	6	10	17	340	1560	960

The values in the parentheses show the inhibitory concentration (IC_{50} in nM) of TE_0 to elicit the half maximal responses in each assay. The values of TE_{12} and DTE_{12} are % potency relative to TE_0 (=100%).

Table 2. Opioid Activities of Monomeric and Dimeric Pentapeptide Enkephalin Analogs in Muscle Assays and Rat Brain Binding Assays

Peptide	Relative μ-activity			Relative δ-activity		
	GPI	^{3}H-NAL	^{3}H-DAGO	MVD	^{3}H-DADLE	^{3}H-DSLET
PE_0	100	100	100	100	100	100
	(29)	(0.64)	(1.2)	(0.31)	(2.0)	(0.94)
PE_2	112	76	64	146	128	46
DPE_2	60	75	124	2770	606	224

The values in the parentheses show the inhibitory concentration (IC_{50} in nM) of PE_0 to elicit the half maximal responses in each assay. The values of PE_2 and DPE_2 are % potency relative to PE_0 (=100%).

^{3}H-DADLE, and the association rate was about 2.5 times larger.[6] The finding in the present study together with those previously obtained strongly suggests that dimers can serve as bivalent ligands to bridge simultaneously the two binding sites of δ receptors. Although there has been no structural elucidation of receptors yet, some different modes of interactions might be possible between dimers and δ receptors. For instance, for bivalent interaction of enkephalin dimers, δ receptors should fulfill several conditions as follows. (1) δ receptors may microaggregate to form a cluster and two receptor molecules are in a close proximity. (2) δ receptor may have the subunit structure consisting of at least two proteins each containing a ligand binding site. Bivalent structure has been shown, for example, for acetylcholine receptor[11] and insulin receptor.[12] (3) δ receptor may have a subsite for ligand-binding in addition to the intrinsic binding site in its single molecule. Figure 3 depicts the mode of interaction of enkephalin dimers with δ receptors. For clarification of the nature of δ receptors, enkephalin dimers may become one of promising probes.

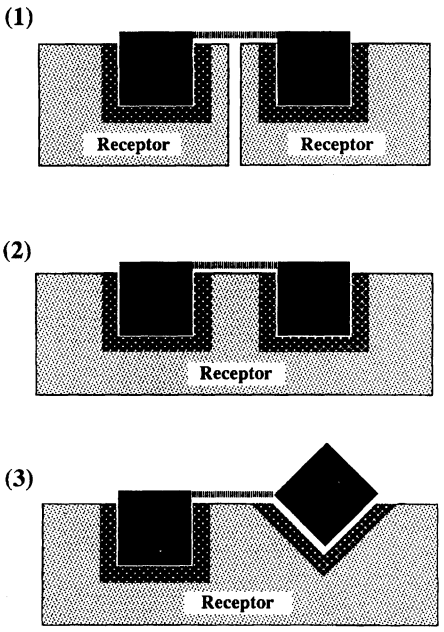

Fig. 3. Possible mode of interactions of enkephalin dimers with δ-opioid receptors. (1) Dimer bridges two receptors simultaneously. (2) Dimer binds to both of binding sites of two independent subunit proteins in a receptor. (3) Dimer interacts with the subbinding site in addition to the major binding site.

Experimental

Synthesis. High-performance thin-layer chromatography (HP-TLC) was carried out on Silica Gel G (Merck, Frankfurt) with the following systems (v/v): R_f^1, $CHCl_3$–MeOH (9:1); R_f^2, *n*-BuOH–AcOH–H_2O (4:1:2, organic phase); R_f^3, *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2); R_f^4, 0.1% AcOH–*n*-BuOH–pyridine (11:5:3, organic phase). Optical rotations were measured with a Perkin–Elmer Model 241MC polarimeter. Melting points were measured using a 6427-H10 Thomas-Hoover melting point apparatus and were uncorrected. HPLC was carried out on a column (4×150 mm) of Hitachi #3063 C18 gel at a flow rate of 1.0 ml min^{-1} by a linear gradient of 5—95% MeOH in 0.1 M NH_4OAc (1 M=1 mol dm^{-3}) over 60 min. Retention time is shown by RT values in minute.

Boc–Phe–NH–$(CH_2)_{11}$–CH_3 (I): To a solution of

Boc–Phe–OH (531 mg, 2 mmol) and dodecylamine (371 mg, 2 mmol) in DMF (10 ml) were added EDC·HCl (422 mg, 2.2 mmol) and HOBt (368 mg, 2.4 mmol) at 0°C. The reaction mixture was stirred at 0°C for 2 h and at room temperature overnight. After evaporation in vacuo, the residue was treated with cold water to solidify. Obtained solid was crashed well and washed successively with 4% $NaHCO_3$, H_2O, 10% citric acid, and H_2O, and dried. Recrystallization was carried out by using a combination of solvents DMF–ether–petroleum ether: Yield, 596 mg (72%); mp 92—93°C; $[\alpha]_D^{20}$ −2.5° (*c* 0.5, DMF); R_f^1 0.93. Found: C, 71.69; H, 10.11; N, 6.29%. Calcd for $C_{26}H_{44}O_3N_2 \cdot 1/4H_2O$: C, 71.43; H, 10.26; N, 6.41%.

Boc–Tyr–D-Ala–Gly–Phe–NH–$(CH_2)_{11}$–CH_3 (II): Compound **I** (206 mg, 0.47 mmol) was dissolved in trifluoroacetic acid (TFA) (3 ml) at 0°C for 30 min. After evaporation, the residue was evaporated to dryness. The resulting H–Phe–NH–$(CH_2)_{11}$–CH_3·TFA (211 mg, 100%) was dissolved in DMF (7 ml) together with Boc–Tyr–D-Ala–Gly–OH[3] (205 mg, 0.5 mmol) and Et_3N (70 μl, 0.5 mmol). To this solution were added EDC·HCl (105 mg, 0.55 mmol) and HOBt (92 mg, 0.6 mmol) at 0°C. After evaporation, the residue was washed with 4% $NaHCO_3$, 10% citric acid, and H_2O, and dried over Na_2SO_4. The oily material after evaporation was purified on a silica gel (silicic acid, 100—300 mesh; Sigma, St. Louis, USA) column (2.2×26 cm) eluted with a solvent mixture of $CHCl_3$-MeOH-AcOH (95 : 5 : 1, v/v). The fractions containing a pure material were collected and evaporated, and the residue was crystallized from EtOAc–ether–pet. ether: Yield, 228 mg (63%); mp 139—141°C; $[\alpha]_D^{20}$ −14.8° (*c* 0.5, DMF); R_f^1 0.59. Found: C, 66.37; H, 8.53; N, 9.46%. Calcd for $C_{40}H_{61}O_7N_5$: C, 66.37; H, 8.48; N, 9.68%.

H–Tyr–D-Ala–Gly–Phe–NH–$(CH_2)_{11}$–CH_3·AcOH (III): Compound **II** (71 mg, 0.1 mmol) was treated with TFA (1 ml) at 0°C for 30 min. After evaporation of TFA, the residue was dissolved in a small amount of 30% AcOH and the solution was put on a column (2.2×110 cm) of Bio-Gel P-2 (200—400 mesh) eluted with 30% AcOH. The fractions containing a pure product were pooled and lyophilized: Yield, 62 mg (91%); mp 126—128°C; $[\alpha]_D^{20}$ +25.8° (*c* 0.5, 95% AcOH); R_f^2 0.48, R_f^3 0.84, R_f^4 0.85; RT 40.

Boc–Phe–Leu–NH–CH_2–CH_3 (IV): This compound was prepared from Boc–Phe–Leu–OH (757 mg, 2 mmol) and ethylamine (116 μl, 2 mmol) by the EDC–HOBt method as described. The reaction mixture was evaporated in vacuo, and the residue was dissolved in EtOAc. The solution was washed with 4% $NaHCO_3$, 10% citric acid and water, and dried over Na_2SO_4. After evaporation, the residue was crystallized from EtOAc–ether–pet. ether: Yield, 730 mg (90%); mp 166—167°C; $[\alpha]_D^{20}$ −18.9° (*c* 0.5, DMF); R_f^1 0.88. Found: C, 65.17; H, 8.70; N, 10.60%. Calcd for $C_{22}H_{35}O_4N_2$: C, 65.16; H, 8.70; N, 10.36%.

Boc–Tyr–D-Ala–Gly–Phe–Leu–NH–CH_2–CH_3 (V): Compound **IV** (203 mg, 0.5 mmol) was dissolved in TFA (3 ml) at 0°C for 30 min. After evaporation, the residue was evaporated to dryness. The resulting H–Phe–Leu–NH–CH_2–CH_3·TFA (219 mg, 100%) was dissolved in DMF (7 ml) together with Boc–Tyr–D-Ala–Gly–OH (205 mg, 0.5 mmol) and Et_3N (70 μl, 0.5 mmol). This solution was treated as described for compound **III**, and purification was carried out on a silica gel (silicic acid, 100—300 mesh) column (2.2×27 cm) eluted with a solvent mixture of $CHCl_3$–MeOH–AcOH (95 : 5 : 1, v/v). The fractions containing a pure material were collected and evaporated, and the residue was crystallized from EtOAc–ether–pet. ether: Yield, 256 mg (74%); mp 198—200°C; $[\alpha]_D^{20}$ −14.5° (*c* 0.5, DMF); R_f^1 0.46. Found: C, 60.56; H, 7.47; N, 12.04%. Calcd for $C_{36}H_{52}O_8N_6 \cdot H_2O$: C, 60.48; H, 7.61; N, 11.76%.

H–Tyr–D-Ala–Gly–Phe–Leu–NH–CH_2–CH_3·AcOH (VI): Compound **V** (70 mg, 0.1 mmol) was treated with TFA (1 ml) at 0°C for 30 min. After evaporation of TFA, the residue was dissolved in a small amount of 30% AcOH and the solution was put on a column (2.2×110 cm) of Bio-Gel P-2 eluted with 30% AcOH. The fractions containing a pure product were pooled and lyophilized: Yield, 61 mg (92%); mp 131—133°C; $[\alpha]_D^{20}$ +15.5°; (*c* 0.5, 95% AcOH); R_f^2 0.39, R_f^3 0.81, R_f^4 0.84; RT 41.

Receptor Biding Assays. Receptor binding assays by using rat brain membrane preparations were carried out essentially as described previously.[13] ^{3}H-DAGO (1.4 TBq/mmol, New England Nuclear) and ^{3}H-NAL (1.4 TBq/mmol, New England Nuclear) were utilized as tracers specific for μ receptors at the final concentration of 0.25 nM. As δ receptor specific tracers, ^{3}H-DADLE (1.5 TBq/mmol, New England Nuclear) and ^{3}H-DSLET (1.5 TBq/mmol, New England Nuclear) were used. Incubations were carried out at 25°C for 60 min in 50 mM Tris-HCl buffer (pH 7.5) containing bacitracin (100 μg ml^{-1}) as an enzyme inhibitor. Dose-response curves were constructed using 7—10 dose levels in duplicate. The data were utilized to construct the least-square estimates of the logistic curves.[14]

Smooth Muscle Assays. For GPI assay, guinea pigs (males, 300—400 g) were decapitated and the longitudinal muscle of the ileum was prepared.[1] The strips were mounted in a 5 ml bath in Krebs-Ringer bicarbonate solution and field-stimulated (60 V, 0.5 ms, 0.1 Hz). For MVD assay, vas deferens from NMRI mice (25—30 mg) were prepared[1] and hung as described above. Data were expressed as percentage of the control responses to electrically evoked twitch tension before addition of the peptides to the bath. The concentration required to produce a half-maximal effect (EC_{50}) was calculated from concentration-response curves.

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9) Abbreviations: DADLE, [D-Ala^2, D-Leu^5]enkephalin; DAGO, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin; DSLET, [D-Ser^2, Leu^5]enkephalyl-Thr^6; DPE_2, dimeric pentapeptide enkephalin crosslinked by ethylenediamine; DTE_{12}, dimeric tetrapeptide enkephalin crosslinked 1, 12-diaminododecane; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; GPI, guinea pig ileum; MVD, mouse vas deferens; NAL, naloxone; PE_0, pentapeptide enkephalin amide; PE_2, pentapeptide enkephalin ethylamide; TE_0, tetrapeptide enkephalin amide; TE_{12}, tetrapeptide enkephalin dodecylamide; and TFA, trifluoroacetic acid.

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