Journal of Peptide Science, Vol. 2, 245–251 (1996)

Multivalent Ligand System Carrying Enkephalin and Neurotensin Coimmobilized on Liposomes

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Received 6 February 1996
Accepted 18 March 1996

Abstract: A multivalent ligand system was constructed by coimmobilization of two kinds of peptide ligands, enkephalin and neurotensin derivatives having a dioctadecyl group, on dimyristoylphosphatidylcholine (DMPC) liposomes. The enkephalin derivatives are Tyr-D-Ala-Gly-Trp-Leu-(Sar-Sar-Pro)$_n$-[N($C_{18}H_{37}$)$_2$] (Enk3nD, $n=0$, 1, 2), where a dioctadecyl group was connected to the C-terminal side of enkephalin directly or through a hydrophilic and flexible spacer chain of different lengths. The neurotensin derivatives are Ac-Glu[N($C_{18}H_{37}$)$_2$]-(Sar-Sar-Pro)$_n$-Arg-Arg-Pro-Tyr-Ile-Leu-OH (D3nNT, $n=0$, 1, 2, 3). The derivatives were spontaneously immobilized on DMPC liposomes by overnight incubation. The receptor affinity of the enkephalin derivatives became significantly higher upon immobilization on liposomes. The highest affinity was obtained for the δ receptor by Enk6D immobilized on DMPC liposomes. This affinity is higher than that of enkephalinamide. Neurotensin derivatives coimmobilized with large amounts of Enk3D on DMPC liposomes show higher affinity than the neurotensin derivatives immobilized alone. The effect of Enk3D on the receptor affinity of the coimmobilized neurotensin derivative disappeared by the addition of [Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGO). Therefore, the receptor affinity of a peptide hormone is altered by immobilization on DMPC liposomes and by coimmobilization with other peptide hormones. It was confirmed by fluorescent microscopy that the multivalent ligand system binds to receptors without release of the bound ligands from DMPC liposomes.

Keywords: enkephalin; neurotensin; opioid receptor affinity; multivalent ligand; liposome; fluorescence microscopy

INTRODUCTION

Chemical signals conveyed by peptide hormones are converted to intracellular signals via various transduction cascades, depending on the type of the target receptors on cell membranes [1]. Because these cascades interact with each other, one signalling process will be affected by other signalling processes in a feed-back or feed-forward manner [2]. In other words, a chemical signal may be enhanced by the simultaneous activation of other cascades. A typical example is the GABA receptor, which consists of a supramolecular structure including GABA and benzodiazepine recognition sites [3]. Based on the structure of the receptor, benzodiazepine modulates the GABA activity through an allosteric effect. Another example is the enhancement by [Leu]enkephalin of the analgesic activity of morphine at low concentration, suggesting that the δ receptor inter-

Abbreviations: BOP, benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate; D3nNT, Ac-Glu[N($C_{18}H_{37}$)$_2$]-(S'S'P)$_n$-RRPYIL ($n=0$, 1, 2, 3); DAGO, [Ala^2, MePhe^4, Gly-ol^5]-enkephalin; DCC, dicyclohexylcarbodiimide; DPDPE, [D-penicillamine^2, D-penicillamine^5]-enkephalin; DMPC, dimyristoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGTA, ethylene glycol bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; Enk, enkephalin; Enk3nD, YaGWL-(S'S'P)$_n$- [N($C_{18}H_{37}$)$_2$] ($n=0$, 1, 2). For, formyl group; GABA, γ-aminobutyric acid; HAT, a mixture of hypoxanthine, aminopterin and thymidine (10 mM/0.1 mM/1.6 mM); NT, neurotensin; NT 8-13 fragment, neurotensin 8–13; PBS(−), phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions; Rho-DPPE, rhodamine-labelled dipalmitoylphosphatidylethanolamine; S', sarcosine; Tes, *N*-tris(hydrodymethyl)-2-aminoethane-sulphonic acid.

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CCC 1075-2617/96/040245-07

acts with the μ receptor to affect the signalling cascade of morphine [4, 5].

There have been several papers reporting the importance of receptor–receptor interactions during the signal transduction within the cell membrane. It has been reported that binding to the receptor of growth factors such as EGF or insulin induces dimerization of the receptors [6, 7]. The EGF receptors are activated by crosslinking with monoclonal antibody [8]. Opioid receptors in neuroblastoma cells have been shown to form clusters upon binding agonists, but the relationship between the cluster formation and the action of opioid peptides remains to be solved [9]. Elucidation of receptor–receptor interactions is considered to be the key factor for understanding the mechanism of signal transduction systems [2].

Multivalent ligand systems seem to be effective in inducing receptor–receptor interaction by crosslinking. Several multivalent ligand systems have been described such as α-melanophore-stimulating hormone (α-MSH) immobilized on tobacco mosaic virus [10], morphine connected to a Sepharose gel [11], and enkephalin bound to poly(Lys) [12] or dextran [13]. However, in the last three cases, where the ligands are bound to a polymer chain, a fraction of the ligands are not easily accessible to receptors in the cell membrane. In order to avoid the drawback of polymer chains as carriers of multiple ligands, liposomes were used [14]. Enkephalin was connected to a lipid and the enkephalin/lipid conjugate formed liposomes carrying enkephalins on the surface. Although the monomeric ligand derivative showed a low receptor affinity, the enkephalin-immobilized liposomes showed a high affinity comparable to enkaphalinamide. The higher affinity is explained by multisite interaction between peptide-containing liposomes and receptors of the cell membranes.

In the present study, enkephalin and neurotensin were coimmobilized on DMPC liposomes to investigate whether the receptor affinity of one ligand is affected by the other one on the same liposome.

MATERIALS AND METHODS

Materials

Protected amino acids were purchased from Kokusan Chemical Works, Ltd, Japan. Enkephalinamide, DAGO, neurotensin and DMPC were purchased from Sigma Chemical Co., USA. All of the ^{3}H-labelled peptides, neurotensin, DAGO and DPDPE, were obtained from DuPont/NEN Research Products,

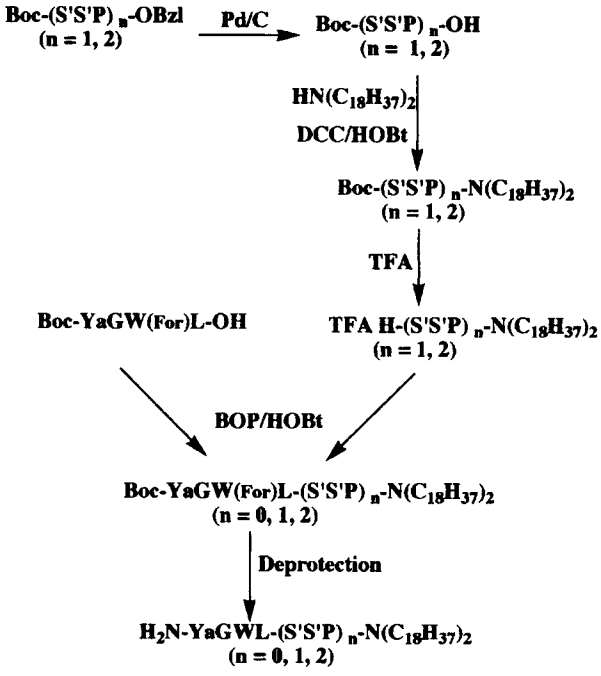

Figure 1 Synthetic scheme of the enkephalin derivatives.

Co., USA. The GF/C glass filter used for binding assays was purchased from Whatman, Ltd, UK.

Syntheses

Synthesis of the neurotensin derivatives, Ac-Glu[N($C_{18}H_{37}$)$_2$]-(Sar-Sar-Pro)$_n$-Arg-Arg-Pro-Tyr-Ile-Leu-OH (D3nNT, n=0, 1, 2, 3), will be reported elsewhere [15]. The enkephalin derivatives, Tyr-D-Ala-Gly-Trp-Leu-(Sar-Sar-Pro)$_n$-[N($C_{18}H_{37}$)$_2$] (Enk3D, n=0, 1, 2), were synthesized by a conventional liquid-phase method except for the enkephalin part which was prepared by solid-phase synthesis using an oxime resin developed by Kaiser *et al.* [16]. The synthetic scheme is shown in Figure 1. All intermediates and final products were identified by ^{1}H-NMR, and the purity was checked by TLC. Analytical TLC was performed on Merck silica gel 60 F_{254} aluminium plates with detection by UV light and/or the ninhydrin test. The solvent systems of TLC were as follows: (I), $CHCl_3$/methanol/ammonia water = 65/25/5 v/v/v; (II) *n*-butanol/acetic acid/water = 10/1/3 v/v/v; (III) *n*-butanol/acetic acid/pyridine/water = 15/3/9/12 v/v/v/v.

In the solid-phase synthesis, tryptophan was protected by the formyl group. The elongation of the peptide chain was carried out by using BOP and

HOBt as coupling reagents. *N*-Hydroxypiperidine was used to cleave the pentapeptide from the resin, and the product was treated with zinc powder in 90% acetic acid. Boc-YaGW(For)L-OH was purified on a Sephadex LH20 column run with DMF, followed by silica gel chromatography using a mixture of chloroform/methanol = 1/1 v/v. Further purification was carried out on a Sephadex LH20 column using methanol as eluant. The main fraction was collected and evaporated. The residue was treated with diethyl ether to obtain a white solid. TLC: R_F(I) = 0.44, R_F(II) = 0.85; ^{1}H-NMR (270 MHz, methanol, 23 °C) δ 0.85 (m, 6H, Leu $C^{\delta}H_3$), 1.22 (d, 3H, Ala $C^{\beta}H_3$), 1.36 (s, 9H, Boc), 1.6 (m, 3H, Leu $C^{\beta}H_2$, C^{γ} H), 2.7–2.9 (m, 2H, Tyr $C^{\beta}H_2$), 3.1–3.25 (m, 2H, Trp $C^{\beta}H_2$), 3.75–3.9 (m, 2H, Gly $C^{\alpha}H_2$), 4.1–4.75 (m, 4H, Tyr, D-Ala, Trp and Leu C^{α}H), 6.65 and 7.0 (m, 4H, Tyr ϕ), 7.0–7.7 (m, 5H, Trp ϕ), 8.25 (s, 1H, Trp CHO).

The hydrophilic spacer chain, Boc-(Sar-Sar-Pro)$_n$-OBzl (n = 1, 2), was synthesized by using DCC and HOBt as coupling reagents. The coupling of dioctadecylamine with enkephalin or neurotensin peptide was carried out by using BOP and HOBt. The products were purified on an LH-20 column using methanol as eluant. TLC: Boc-YaGW(For)L-N($C_{18}H_{37}$)$_2$, R_F(I) = 0.83, R_F(II) = 0.99; Boc-YaGW-(For)L-S'S'P-N($C_{18}H_{37}$)$_2$, R_F(I) = 0.88, R_F(II) = 0.79; Boc-YaGW(For)L-(S'S'P)$_2$-N($C_{18}H_{37}$)$_2$, R_F(I) = 0.75, R_F(II) = 0.63.

The formyl group was removed by hydrazine treatment [17]. The Boc group was detached by TFA treatment. The product was purified by a Sephadex LH-20 column using methanol as eluant, and purified further by a preparative HPLC using a reverse-phase column. The product was lyophilized. The conditions of HPLC: column, 5C18-AR (10 × 250, Nakalai Tesque, Inc., Japan); flow rate, 3 ml/min; CH_3CN/TFA 100/0.05 v/v.

Enk0D: TLC, R_F(I) = 0.92, R_F(II) = 0.85, R_F(III) = 0.92; HPLC, 10.3 min, FAB-MS(MH)$^+$ 1113; ^{1}H-NMR (270 MHz, $CDCl_3$, 23 °C) δ 0.85 (m, 12H, Leu $C^{\delta}H_3$ and acyl chains (CH_3), 1.26 (b, 67H, Ala $C^{\beta}H_3$ and acyl chains CH_2), 1.35–1.60 (b, 7H, Leu $C^{\beta}H_2$, C^{γ} H and acyl chains $C^{\beta}H_2$), 2.7–2.9 (b, 2H, Tyr $C^{\beta}H_2$), 3.1–3.25 (b, 2H, Trp $C^{\beta}H_2$), 3.75–3.9 (b, 2H, Gly $C^{\alpha}H_2$), 4.1–4.75 (b, 4H, Tyr, D-Ala, Trp and Leu $C^{\alpha H}$), 6.65–7.0 (b, 4H, Tyr ϕ), 7.0–7.7 (b, 9H, Trp ϕ, D-Ala, Gly, Trp, Leu, NH).

Enk3D: TLC, R_F(I) = 0.92, R_F(II) = 0.42, R_F(III) = 0.82, HPLC, 9.5 min, FAB-MS(MH)$^+$ 1352; ^{1}H-NMR (270 MHz, $CDCl_3$, 23 °C) δ 0.85 (m, 12H, Leu $C^{\delta}H_3$ and acyl chains CH_3), 1.26 (b, 67H, Ala $C^{\beta}H_3$ and acyl chains CH_2), 1.35–2.1 (b, 11H, Leu $C^{\beta}H_2$, C^{γ} H, acyl chains $C^{\beta}H_2$, and Pro $C^{\beta}H_2$, $C^{\gamma}H_2$), 2.1–3.1 (b, 8H, Tyr $C^{\beta}H_2$ and Sar N-CH_3), 3.1–3.25 (b, 2H, Trp $C^{\beta}H_2$), 3.25–3.9 (b, 4H, Gly $C^{\alpha}H_2$ and Pro $C^{\delta}H_2$), 4.1–4.75 (b, 5H, Tyr, D-Ala, Trp, Leu, Pro C^{α}H), 6.65–7.6 (b, 13H, Tyr ϕ, Trp ϕ, D-Ala, Gly, Trp, Leu NH).

Enk6D: TLC, R_F(I) = 0.91, R_F(II) = 0.21, R_F(III) = 0.76, HPLC, 8.9 min; FAB-MS(MH)$^+$ 1591; ^{1}H-NMR (270 MHz, $CDCl_3$, 23 °C) δ 0.85 (m, 12H, Leu $C^{\delta}H_3$ and acyl chains CH_3), 1.26 (b, 67H, Ala $C^{\beta}H_3$ and acyl chains CH_2), 1.35–2.15 (b, 15H, Leu $C^{\beta}H_2$, C^{γ} H, acyl chains $C^{\beta}H_2$, and Pro $C^{\beta}H_2$, $C^{\gamma}H_2$), 2.7–3.1 (b, 14H, Tyr $C^{\beta}H_2$ and Sar N-CH_3), 3.1–3.25 (b, 2H, Trp $C^{\beta}H_2$), 3.25–3.6 (b, 6H, Gly $C^{\alpha}H_2$ and Pro $C^{\delta}H_2$), 3.8–4.75 (b, 6H, Tyr, D-Ala, Trp, Leu, Pro C^{α}H), 6.6– 7.6 (b, 13H, Tyr ϕ, Trp ϕ, D-Ala, Gly, Trp, Leu NH).

Incorporation of Enkephalin Derivatives into DMPC Liposomes

DMPC liposomes were prepared by a sonication method in a TRIS buffer (10 mM, pH 7.4, EDTA 0.1 mM). An aliquot of the enkephalin derivatives in ethanol was added to DMPC liposomes. The final concentrations of DMPC and the peptides were 4.0 mM and 20 μM, respectively. The enkephalin derivatives were also dispersed in a TRIS buffer solution. The dispersion was incubated at 30 °C overnight, followed by an 1 h incubation at 4 °C. After centrifugation at 15,000*g* for 15 min, the supernatant and the precipitate dissolved in methanol were subjected to fluorescence measurement. Excitation and monitoring wavelengths were 280 nm and 336 nm, respectively.

Receptor Affinity

Enkephalin and neurotensin were chosen as ligands. These peptides act as neurotransmitters and both receptors exist in bovine brain homogenate and neuroblastoma cells [18, 19]. Therefore, the receptor affinity of the peptides was determined by competitive inhibition of ^{3}H-labelled ligand binding to bovine-brain homogenate [20] by the peptides to be assessed. The receptor affinity was evaluated as IC_{50}, which is the ligand concentration necessary to occupy half the binding sites of the receptor. [^{3}H]DPDPE and [^{3}H]DAGO were used as ligands competing for the δ and μ receptors, respectively. Two types of membrane homogenates were prepared. One of them was prepared by a conventional method, which was a membrane fraction precipitated by

Table 1 The Incorporation of Enkephalin Derivatives to DMPC Liposomes. The Numerical Values Represent Fluorescence Intensity (Arbitrary Unit)

	In TRIS buffer			With liposomes		
	Before centrifugation	Supernatant	Precipitate[a]	Before centrifugation	Supernatant	Precipitate[a]
Enk0D	1.86	0	17.8	38.8	38.2	0
Enk3D	8.43	0	18.2	37.7	39.5	0
Enk6D	9.4	0	20.4	37.6	36.4	0

[a] The precipitates were dissolved in methanol of the same volume as the buffer solution and the fluorescence intensity of the solution was measured.

30,000*g* [21] and was used for the assay of opioid receptor affinity. The other was a membrane fraction precipitated between 1000*g* and 27,000*g*, which was rich in neurotensin receptors [22] and was used for the assay of neurotensin receptor affinity.

The assay buffer was Tes-KOH buffer (10 mM, pH 7.5) containing EGTA-K^+ (1 mM), bacitracin (0.01%), soybean trypsin inhibitor (0.002 wt%), benzamidine-HCl (1 mM), 1,10-phenanthroline (1 mM), bestatin (10 μM) and bovine serum albumin (0.02 wt%). A test tube with a total volume of 300 μl containing the membrane (0.5–1 mg/ml proteins), 2.5 nM ^{3}H-labelled ligand, and different concentrations of the peptides were used in the binding experiments. The suspension was incubated at 20 °C for 1 h. The binding was terminated by the addition of 2 ml of ice-cold TRIS buffer solution (50 mM, pH 7.4) followed by filtration under reduced pressure through Whatman GF/C glass filters. Tubes and filters were washed three times with 2 ml of ice-cold TRIS buffer solution. The filters were immersed in 5 ml of Clear-sol (Nakalai Tesque Company, Japan) scintillation solution, and the radioactivity was counted on an LSC-1000 β Counter (Aloka, Co., USA). Nonspecific binding was estimated in parallel experiments in the presence of an excess amount (1 μM) of unlabelled ligand (neurotensin for neurotensin receptor, [Leu]enkephalin for the δ and the μ receptors).

Observation of Cells by Fluorescence Microscopy

Neuroblastoma NG108-15 cells were kindly provided by Dr Haruhiro Higashida (Department of Medicine, Kanazawa University, Kanazawa, Japan). Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Gibco Co., USA) supplemented with 5% fetal calf serum and 1% HAT (Gibco, 0.1 mM hypoxanthine, 1 μM aminopterin and 16 μM thymidine), in humidified air containing 10% CO_2. The cells were detached from the culture dishes by incubation with Dulbecco's PBS(−) (Nissui Pharmaceutical Co., Ltd, Japan) buffer at 37 °C for 5 min, and the suspension was centrifuged at 1000*g* for 10 min. The cells were suspended in DMEM containing 5% fetal calf serum and allowed to grow for two days under the standard culture conditions described above. Then, the medium was removed from the plates, and the cells were incubated in 3 mM DMEM (pH 7.4) containing the peptide derivatives (0.6 μM) immobilized on DMPC liposomes ([DMPC] = 0.12 mM), which were labelled with 5 mol% of Rho-DPPE. The incubation was carried out at 37 °C for 1 or 1.5 h, and stopped by removing the medium and cooling. The cells were observed with a BH-2 optical and fluorescent microscope (Olympus Co., Japan).

RESULTS AND DISCUSSION

Uptake of Enkephalin Derivatives by DMPC Liposomes

The neurotensin derivatives have been reported to be immobilized on DMPC liposomes by overnight incubation [5]. Uptake of the enkephalin derivatives to DMPC liposomes was investigated, and the results are shown in Table 1. Enk0D in a TRIS buffer solution was only weakly fluorescent immediately after the preparation of solution. Upon incubation overnight and centrifugation of the solution, the supernatant contained no fluorescent components, indicating that nearly all peptides precipitated. Obviously, Enk0D is insoluble in buffer solution and aggregates because of hydrophobic interactions. On the other hand, Enk3D and Enk6D in a TRIS buffer solution were moderately fluorescent immedi-

ately after preparation of the solution. Enk3D and Enk6D are more soluble in the buffer solution than Enk0D owing to the presence of the hydrophilic spacer chain. However, upon incubation overnight and centrifugation, the peptides were not found in the supernatant but in the precipitate, indicating aggregation of these peptides, too (Table 1).

In the presence of DMPC liposomes, the fluorescence intensity of the dispersion of the enkephalin derivatives did not change before and after the overnight incubation and centrifugation (Table 1). Since DMPC liposomes are small unilamellar vesicles, they do not precipitate during the centrifugation. Therefore, all enkephalin derivatives are quantitatively taken up by the DMPC liposomes.

When the peptides were incubated with DMPC liposomes for 3 h, the fractions of the peptides taken up by DMPC liposomes were 87% for Enk0D, 69% for Enk3D and 65% for Enk6D, indicating that incubation for 3 h is not enough for complete uptake. The rate of uptake decreases in the order of Enk0D > Enk3D > Enk6D, which may be correlated with the solubility of the enkephalin derivatives in the buffer solution. The lower the solubility of peptide in a buffer solution, the faster the uptake of the liposomes.

Affinity of Enkephalin Derivatives for the Receptor

Enkephalin connected to DPPE [14] and a neurotensin fragment having a dioctadecyl group [15] have been reported to show an increased receptor affinity after immobilization on liposomes. The receptor affinity was dependent on the molar ratio of lipid to peptide, and the highest affinity was obtained when the lipid/peptide molar ratio was 200–400 [14, 15].

The enkephalin derivatives synthesized in the present study were immobilized on DMPC liposomes, and their affinity for the μ receptor in bovine brain membranes was determined (Table 2). The receptor affinity was very low when the derivatives were suspended in a TRIS buffer solution. However, the affinity was considerably higher after immobilization on DMPC liposomes. The affinity is dependent on the length of the spacer chain connecting the between enkephalin part and a dioctadecyl group, and increases in the order of Enk0D < Enk3D < Enk6D. This result may be explained in terms of steric hindrance. The peptide segment of the enkephalin derivative with a long and hydrophilic spacer should protrude into the aqueous phase. In the case of Enk6D, the binding of the peptide segment immobilized on the liposome to the receptors on the cell membrane should be free from severe steric hindrance thus resulting in high receptor affinity. However, the affinity of Enk6D on liposomes is still only one-eighth of enkephalinamide in solution.

Table 2 Inhibition of [^{3}H]DAGO Binding to Bovine Brain Homogenate by Enkephalin Derivatives Expressed as IC_{50} (nM)

Enkephalin derivatives	Dispersed in TRIS buffer solution	Immobilized on DMPC liposome[a]
Enk0D	> 1000	129 ± 24
Enk3D	> 1000	38 ± 5.0
Enk6D	> 1000	27 ± 7.5
Enk-NH_2	3.3 ± 0.9	

[a] [DMPC]/[ligand] = 200.

Table 3 Inhibition of [^{3}H]DEDPE Binding to Bovine Brain Homogenate by Enkephalin Derivatives Expressed as IC_{50} (nM)

Enkephalin derivatives	Dispersed in TRIS buffer solution	Immobilized on DMPC liposome[a]
Enk0D	> 1000	3.6 ± 1.5
Enk3D	160 ± 68	3.2 ± 0.9
Enk6D	130 ± 53	2.1 ± 0.8
Enk-NH_2	4.0 ± 1.2	

[a] [DMPC]/[ligand] = 200.

Affinity of Enkephalin Derivatives for the δ Receptor

The affinity of the enkephalin derivatives for the δ receptor was also higher after immobilization on DMPC liposomes (Table 3). The highest affinity for the receptor was obtained with Enk6D immobilized on DMPC liposomes, which is superior to enkephalinamide in solution.

The affinity of the immobilized enkephalin derivatives is better for the δ receptor and higher than that for the μ receptor. According to the membrane compartment concept of Schwyzer [23], the binding site of the μ receptor is located in the charged layer of the bilayer membrane and that of the δ receptor is exposed to the aqueous phase. The enkephalin segment supported on a liposome may gain easier access to the δ receptor than to the μ receptor because of lower steric hindrance.

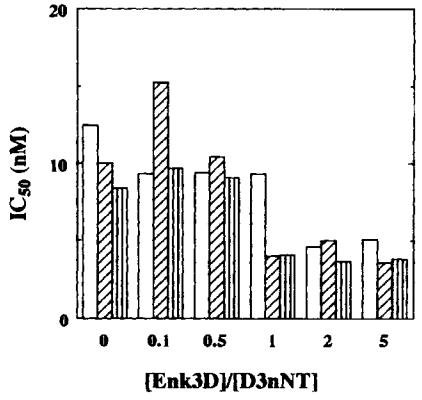

Figure 2 The effect of Enk3D coimmobilization on DMPC liposomes on inhibition of [^{3}H]neurotensin binding to the bovine brain homogenate by the neurotensin derivatives, D3NT (□), D6NT (▨) and D9NT (▥), which are immobilized on DMPC liposomes. The molar ratio of DMPC and the neurotensin derivatives was kept at 200.

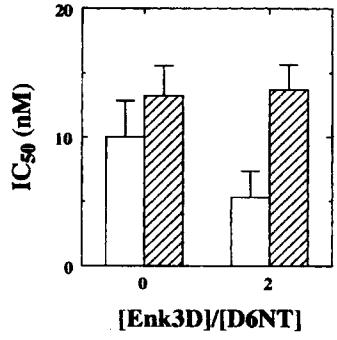

Figure 3 The affinity for neurotensin receptor of D6NT immobilized alone or coimmobilized with Enk3D on DMPC liposomes in the absence (□) and presence (▨) of DAGO (1 μM). [DMPC]/[D6NT] = 200.

Neurotensin Derivative and Enkephalin Derivative Coimmobilized on DMPC Liposomes

The high receptor affinity of the peptide ligand immobilized on DMPC liposomes could be explained by multisite interaction between the ligands immobilized on liposomes and the receptors existing on the cell membrane [15]. The affinity for the neurotensin receptor was investigated with varying molar ratios of Enk3D and neurotensin derivatives (D3nNT) in the liposomes (Figure 2). The affinity was higher after coimmobilization of D3NT with Enk3D when the molar ratio of Enk3D/D3nNT was unity or higher.

D6NT coimmobilized with Enk3D on DMPC liposomes showed a higher affinity for the neurotensin receptor than without Enk3D. This effect disappeared in the presence of DAGO, which is the agonist for the μ receptor and also for the δ receptor at high concentrations (Figure 3). It is concluded that the effect of Enk3D is induced by binding of Enk3D to its specific receptors, which influences the receptor binding of D6NT coimmobilized on the liposome.

Observation by Fluorescence Microscopy

NG108-15 cells have been reported to possess neurotensin receptors as well as δ receptors [24–26]. The binding of the peptide-carrying liposomes to receptors in NG108-15 cells was investigated by fluorescence microscopy. Enk6D immobilized on DMPC liposomes containing 5 mol% Rho-DPPE was incubated with NG108-15 for 1.5 h (Figure 4(A)). The cells were detected by rhodamine fluorescence, indicating the binding of DMPC liposomes to the cells. The same result was obtained by Enk0D and Enk3D immobolized on DMPC liposomes. Therefore, the enkephalin derivatives bind to the δ receptors as being immobilized on liposomes.

NG108-15 cells were incubated with Enk3D/D6NT containing liposomes. These were found by rhodamine fluorescence to be bound to the cells (Figure 4(B)). The fluorescence became more intensive with time but reached a maximum intensity after 1 h of incubation. The fluorescence intensity of cells incubated with Enk3D/D6NT contaning DMPC liposomes increased faster than that of cells incubated with immobilized Enk3D alone.

CONCLUSIONS

The neurotensine receptor affinity was higher after coimmobilization. The enhanced affinity may be explained in terms of multisite interaction of the liposomes with the cell membranes, in which the enkephalin derivative/opioid receptor and the neurotensin derivative/receptor interactions are simultaneously involved. The rate of binding of the enkephalin/neurotensin liposomes was faster than that of the enkephalin-immobilized liposomes. Our multivalent ligand system consisting of two different ligands coimmobilized on liposomes seems to be effective in simultaneous activation of two types of receptors existing in a localized area within the cell membrane. The multivalent ligand system, in which two kinds of ligands can crosslink their specific receptors efficiently, will provide useful information

on receptor–receptor interactions and open a way to application of chemically modified peptide hormones in the medicinal field.

Acknowledgements

This work was supported in part by a grant from the Nissan Science Foundation.

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