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Receptor affinity of neurotensin message segment immobilized on liposome

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Abstract

Neurotensin derivatives having a dioctadecyl group were synthesized and immobilized on DMPC liposome to construct a multivalent-ligand system. The derivatives are Ac-Glu[N($C_{18}H_{37}$)$_2$]-(Sar-Sar-Pro)$_n$-Arg-Arg-Pro-Tyr-Ile-Leu-OH (D3*n*NT, n = 0,1,2,3), where a dioctadecyl group was connected to the N-terminal side of neurotensin 8–13 fragment directly or through a hydrophilic and flexible spacer chain of different lengths. The derivatives were spontaneously immobilized on DMPC liposome upon incubation overnight. The receptor affinity of the derivatives increased significantly upon immobilization on liposome. The maximum affinity was obtained by D9NT immobilized on DMPC liposome at the molar ratio of DMPC and D9NT of 200. This affinity is slightly better than the neurotensin 8–13 fragment, the message segment of the derivatives. The fluorescent microscopy using rhodamine-labelled liposome revealed that the multivalent-ligand system binds to specific receptors without dissociation of the derivative from DMPC liposome.

Keywords: Neurotensin; Opioid receptor affinity; Multivalent ligand; Liposome; Neurotensin-lipid conjugate; Fluorescence microscopy

1. Introduction

Endogenous peptide hormones are signaling molecules which regulate the activities of the endocrine system and the nervous system. The importance of medicinal application of the peptides will be extensively increasing in near future, because of the high pharmacological efficiency and low toxicity or side effects in administration [1]. Many attempts have been made to reduce the molecular size of peptide hormones to the minimum-essential length for the pharmaceutical activity [2], to increase resistance against biodegradation in vivo [3], and to decrease undesired side effects due to antigenicity [4].

Construction of divalent or multivalent peptidic ligands can be a way to control biological activities of peptide hormones. Divalent ligands are the conjugate of two message segments of the same kind or different kinds of peptide hormones through a spacer chain of different lengths. It has been reported that divalent ligands showed unique receptor selectivity [5] or activated a biologically low-potent peptide toward opioid receptor [6]. These biological effects of bivalent ligands might have arisen from simultaneous binding to two types of receptors which are separated in a distance suitable for cross-linking by the divalent ligand. An example of multivalent ligand has been reported, in which several molecules of α-melanophore-stimulating hormone (α-MSH) are connected to tobacco mosaic virus (TMV) [7]. The multivalent ligand showed an enhanced receptor affinity due to multisite interaction between TMV and receptors in cell membrane. The idea of multisite interaction should be useful for designing peptide conjugate with controlled biological activities [8].

Abbreviations: Ac, acetyl group; Boc, N^{α}-*t*-butyloxycarbonyl; BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; $BzCl_2$, 2,6-dichlorobenzyl; D3*n*NT, Ac-Glu[N($C_{18}H_{37}$)$_2$]-(S′S′P)$_n$-RRPYIL (n = 0,1,2,3); DCC, dicyclohexylcarbodiimide; DMEM, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; DMPC,dimyristoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HAT, a mixture of hypoxanthine, aminopterin and thymidine(10 mM/0.1 mM/1.6 mM); Hepes, *N*-2-hydroxylethylpiperazine-*N′*-2-ethanesulfonic acid; HOBt, *N*-hydroxybenzotriazole; HPLC, high performance liquid chromatography; IC_{50}, the ligand concentration necessary to occupy the half of the binding sites of receptor; MSA, methanesulfonic acid; Mts, 2,4,6-trimethylbenzenesulfonyl; NT, neurotensin; OEt, ethyl ester; PBS(−), phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions; Rho-DPPE, Rhodamine-labelled DPPE; S′, sarcosine; Tes, *N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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We have investigated poly(lysine) [9] and dextran [10] for the carrier of enkephalin molecules. However, the receptor affinity of the multivalent system on the basis of an enkephalin unit was lower than the enkephalin itself, probably due to steric hindrance of the carrier molecule against the interaction of the enkephalin unit with receptors in membrane. On the other hand, a multivalent system using liposome as the carrier, to which enkephalin molecules carrying long alkyl chains were immobilized, showed high receptor affinity because of low steric hindrance of liposome in multisite binding to receptors [11]. The enkephalin derivatives having long alkyl chains were spontaneously incorporated into the liposome by incubation. This state is represented by 'immobilization of ligand on the surface of liposome', although the ligand is allowed a 2-dimensional diffusion.

In the present study, we applied the liposomal multivalent ligand system to neurotensin, which differs from enkephalin in the following points: (i) different signal transduction systems (the signal transmission by cAMP-dependent protein kinase for enkephalin [12] and by protein kinase C for neurotensin [13]), (ii) the high affinities of enkephalin by the N-terminal ammonium group [14], and neurotensin by the C-terminal carboxyl group [15]. Therefore, long alkyl chains were connected to C terminal of enkephalin and to N terminal of neurotensin as anchoring group for immobilization. The effectiveness of multivalent ligand system consisting of peptide hormones immobilized on liposome should be established not only with enkephalin but also with neurotensin.

2. Materials and methods

2.1. Materials

Protected amino acids were purchased from Kokusan Chemical Works, Japan. ^{3}H-labeled neurotensin was obtained from DuPont/NEN Research Products, USA. The GF/C filters used for binding assay were purchased from Whatman, UK. Neurotensin and DMPC were purchased from Sigma, USA. Rhodamine-labelled DPPE was purchased from Molecular Probes, USA. Other chemicals were of highest purity commercially available.

2.2. Synthesis

Syntheses of the neurotensin fragment, the spacer peptides and the neurotensin derivatives were carried out by a conventional liquid-phase method. The synthetic schemes are shown in Fig. 1. All the protected intermediates were purified by an LH20 column using methanol as eluant. All intermediates and final compounds were identified by ^{1}H-NMR, and the purity was checked by TLC. Analytical TLC was performed on Merck silica gel 60 F_{254} aluminum plates with detection by UV light and/or the ninhydrin

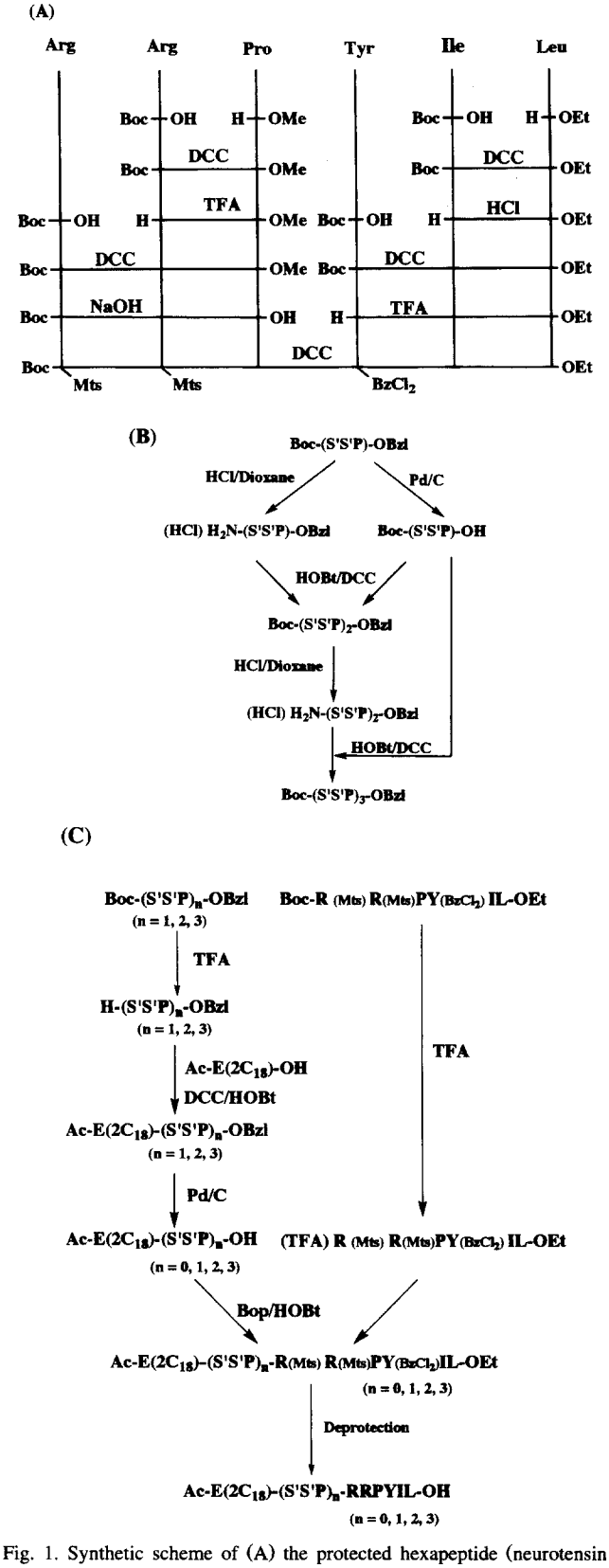

Fig. 1. Synthetic scheme of (A) the protected hexapeptide (neurotensin 8–13 fragment), (B) the hydrophilic oligopeptide (spacer chain), and (C) the neurotensin derivatives.

test. The solvent systems of TLC were as follows: (I), $CHCl_3$/methanol/aqueous ammonia = 65:25:5, v/v/v; (II), n-butanol/acetic acid/water = 10:1:3, v/v/v.

Coupling reactions were carried out by using DCC and HOBt except the coupling between Ac-E($2C_{18}$)-(S'S'P)$_n$-OH and R(Mts)R(Mts)PY($BzCl_2$)IL-OEt, for which BOP and HOBt were used as coupling reagents.

The protecting groups, Mts, Boc and $BzCl_2$ were removed by MSA in the presence of thioanisole, *o*-cresol, and anisole ([MSA]/[thioanisole]/[*o*-cresol]/[anisole] = 0.75:0.25:0.05:0.05, v/v/v/v). The amount of MSA was 50-fold of that of protecting group. Ethyl ester was deprotected by hydrolysis in methanol/dioxane (1:1, v/v) with 2 molar-equivalent of NaOH. The final products were purified by a partition chromatography using a Sephadex G-50 column, which was prepared by an lower phase of a mixture of n-butanol/acetic acid/water (5:1:4, v/v/v). The samples were eluted by the upper phase of the mixture. The main fraction was collected, and purified further by a preparative HPLC using a reverse-phase column. The eluant composition of water/CH_3CN/TFA was changed from 40:60:0.05 to 0:100:0.05 over 20 min. The product was lyophilized.

The purification of the final products of the neurotensin derivatives was carried out by HPLC under following conditions: column, 5C18-AR (10 × 250, Nakalai Tesque, Japan); flow rate, 3 ml/min; gradient, water/CH_3CN/TFA from 40:60:0.05 to 0:100:0.05 over 20 min.

The properties of the neurotensin derivatives (D3*n*NT, $n = 0,1,2,3$) are as follows. Neurotensin 8–13 fragment: TLC (I), $R_f = 0.66$; TLC(II), $R_f = 0.59$; HPLC, 3.6 min. The amino-acid analysis of the product: 1.00 Leu, 1.00 Ile, 0.99 Tyr, 1.00 Pro and 2.00 Arg. D0NT: TLC(I), $R_f = 0.25$; TLC(II), $R_f = 0.21$; HPLC, 13.4 min; ^{1}H-NMR (400 MHz, $CDCl_3$, 23°C) δ 0.85 (m, 18H, Leu $C^\delta H_3$, Ile $C^\gamma H_3$, $C^\delta H_3$ and acyl chains CH_3), 1.24 (s, 64H, acyl chains CH_2), 1.55–1.8 (b, 14H, Ile $C^\beta H$, $C^\gamma H_2$, Leu $C^\beta H_2$, $C^\gamma H$ and Arg $C^\beta H_2$,$C^\gamma H_2$), 1.8–2.3 (b, 11H, acetyl CH_3, Pro and Glu $C^\beta H_2$,$C^\gamma H_2$), 2.5–2.9 (s, 2H, Tyr $C^\beta H_2$), 2.9–3.7 (b, 10H, Pro $C^\delta H_2$, Arg $C^\delta H_2$ and acyl chains $C^\beta H_2$), 4.1–4.6 (b, 7H, Tyr, Arg, Pro, Glu, Ile and Leu $C^\alpha H$), 6.7–7.2 (b, 4H, Tyr φ), 7.5–7.7 (m, 6H, Arg, Leu, Ile,Glu, Tyr NH). D3NT: TLC(I), $R_f = 0.7$; TLC(II), $R_f = 0.37$; HPLC, 12.8 min. ^{1}H-NMR data were the same as those of D9NT except of proton intensities of Sar and Pro residues. D6NT: TLC(I), $R_f = 0.71$; TLC(II), $R_f = 0.42$; HPLC, 12.4 min. ^{1}H-NMR data were the same as those of D9NT except of proton intensities of Sar and Pro residues. D9NT: TLC(I), $R_f = 0.78$; TLC(II), $R_f = 0.39$; HPLC, 12.2 min; ^{1}H-NMR (400 MHz, $CDCl_3$, 23°C) δ 0.85 (m, 18H, Leu $C^\delta H_3$, Ile $C^\gamma H_3$, $C^\delta H_3$ and acyl chains CH_3), 1.24 (s, 64H, acyl chains CH_2), 1.5–2.7 (b, 39H, Ile $C^\beta H$, $C^\gamma H_2$, Leu $C^\beta H_2$, $C^\gamma H$, acetyl CH_3, Arg, Pro and Glu $C^\beta H_2$,$C^\gamma H_2$, Tyr $C^\beta H_2$), 2.9–3.7 (b, 34H, Pro $C^\delta H_2$, Arg $C^\delta H_2$, acyl chains $C^\beta H_2$ and Sar N-CH_3), 3.8–4.6 (b, 22H, Tyr, Arg, Pro, Glu, Ile, Sar and Leu $C^\alpha H$), 6.7–7.2 (b, 4H, Tyr φ), 7.5–7.7 (m, 6H, Arg, Leu, Ile,Glu, Tyr NH).

2.3. Fluorescence depolarization and quenching

An aliquot of a tetrahydrofuran solution of DPH was added to the peptide derivatives in a Tris buffer solution (10 mM, pH 7.4, 0.1 mM EDTA). The solution was incubated for 30 min at prescribed temperature, and fluorescence depolarization was measured by a Hitachi MPF-4 fluorescence spectrophotometer equipped with a homemade apparatus for the depolarization measurement [16], [DPH] = 0.5 μM, [peptide] = 10 μM. Excitation and monitoring wavelengths were 380 and 435 nm, respectively. Fluorescence anisotropy is related with an effective volume of a spherical molecule according to Eq. (1) [17].

$$1/r = (1/r_0)[1 + k_B T\tau/(\eta v)] \quad (1)$$

r, k_B, T, τ, η and v represent fluorescence anisotropy, Boltzman factor, temperature, fluorescence life time, viscosity and the effective volume of the molecule, respectively. The plot of $1/r$ and $(k_B T\tau)/(\eta\tau_0)$, where τ_0 is fluorescence life time at 25°C, gives a straight line, and v is calculated from the slope, $(1/r_0)(\tau_0/v)$.

The peptides (30 μM) were incubated overnight with DMPC liposome ([DMPC] = 6.0 mM) in a Tris buffer solution (10 mM, pH 7.4, 0.1 mM EDTA). Fluorescence spectra of the dispersion were measured with increasing amount of acrylamide at 30°C. Excitation and monitoring wavelengths were 276 and 305 nm, respectively.

2.4. Immobilization of peptides to liposome

DMPC liposome was prepared by a sonication method. A Tris buffer solution (10 mM, pH 7.4, EDTA 0.1 mM) was added to a dry thin film of DMPC, and the mixture was sonicated by a probe-type sonicator at 35°C under N_2 atmosphere. The suspension was centrifuged at 100 000 × *g*. Peptide derivatives were incubated overnight with a prescribed concentration of DMPC liposome at 30°C.

2.5. Distribution of the neurotensin derivatives in DMPC liposome

(i) Examination by a column method: The peptides (30 μM) were incubated overnight with DMPC liposome (6.0 mM) at 30°C, and the suspension was eluted through a Sephadex G-50 column. The peptides were detected by fluorescence at 305 nm. Excitation wavelength was 276 nm. The elution of DMPC liposome was also detected by fluorescence at 305 nm due to light scattering.

(ii) Determination of the amount of peptides not included in liposome: The peptides (20 μM) in the presence or the absence of DMPC liposome (4.0 mM) were incubated at 30°C overnight. The suspension was filtrated

through a Millipore filter by centrifugation at 15000 × *g* (Ultrafree C3TK; cut-off molecular weight, 30000). The amount of peptides was determined before and after the filtration by the fluorescence measurement. Excitation and monitoring wavelengths were 276 and 305 nm, respectively.

2.6. Preparation of bovine-brain homogenate

The receptor affinity of the peptides was determined by using bovine-brain homogenate, which was prepared by a conventional method [18]. All operations were carried out in a cold room. Bovine brain was chopped and suspended in a buffer solution (10 mM Hepes (pH 7.5), 1 mM EDTA, 300 mM sucrose, 0.5 mM dithiothreitol, 1 mM benzamidine-HCl, 0.3 mM phenylmethylsulfonyl fluoride). The suspension was homogenized by a Polytron (Kinematica, Switzerland) and a glass-Teflon homogenizer, and was centrifuged at 1000 × *g* for 12 min. The supernatant obtained was centrifuged at 27000 × *g* for 40 min. The precipitate was resuspended by using a glass-Teflon homogenizer in the above buffer solution without containing sucrose and phenylmethylsulfonyl fluoride. After recentrifugation, the precipitate was resuspended in the latter buffer solution and stored at −85°C.

2.7. Receptor affinity

The buffer solution for assay 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) 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-labeled neurotensin and different concentrations of the neurotensin derivatives was 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 filter. 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, Japan) scintillation solution, and the radioactivity was counted on an LSC-1000 β counter. Nonspecific binding was estimated in parallel experiments in the presence of an excess amount (1 μM) of unlabeled neurotensin. Each assay was performed three times, and the average values and the standard deviations were determined.

2.8. Observation of cells by fluorescence microscope

Neuroblastoma NG108-5 cells were kindly provided by Dr. Haruhiro Higashida (Department of Medicine, Kanazawa University, Kanazawa, Japan). Cells were cultured at 37°C in DMEM (Gibco, USA) supplemented with 5% fetal calf serum and 1% HAT (Gibco, 0.1 mM hypoxanthine, 1 μM aminopterin and 16 μM thymidine), in a humidified atmosphere of air containing 10% CO_2. The cells were detached from the culture dishes by incubation with Dulbecco's PBS(−) (Nissui Pharmaceutical, 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 one day under the standard culture conditions as 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 immobilized in DMPC liposome, which were labelled with 5 mol% of Rho-DPPE. The incubation was carried out at 37°C for 1 h, and stopped by removing the medium and cooling. The cells were observed with a BH-2 optical and fluorescent microscope (Olympus, Japan).

3. Results and discussion

3.1. Neurotensin derivatives in water

A dioctadecyl group was connected to N-terminal side of neurotensin 8–13 fragment through hydrophilic spacer chain of various lengths. The neurotensin derivatives have an amphiphilic structure consisting of the hydrophilic neurotensin fragment and spacer chain and the hydrophobic dioctadecyl group. The amphiphilic structure may promote assembly formation in water, which was investigated by using a hydrophobic fluorescent probe, DPH. DPH does not fluoresce in water, but fluoresces intensively in a hydrophobic environment. In the presence of the neurotensin derivatives in water, DPH fluoresces intensively, indicating incorporation of the probe into a hydrophobic environment formed by the assembly of neurotensin derivatives.

The size of the assembly was evaluated by measuring fluorescence depolarization of DPH incorporated in the assembly, which is related to the Brownian motion of the assembly in water. Fluorescence depolarization of DPH in the peptide assembly was measured at varying temperatures from 15°C to 45°C. In the cases of D0NT, D3NT, and D6NT, fluorescence intensity of DPH became significantly weak with rising temperature, resulting ultimately in a large fluctuation of fluorescence depolarization. It is considered that D0NT, D3NT, and D6NT form unstable aggregate in water. On the other hand, fluorescence depolarization of DPH incorporated in D9NT assembly was measured to a quantitative level. The plot of $1/r$ vs. $(k_B T\tau)/(\eta\tau_0)$ gave a straight line (Fig. 2). Assuming that the peptide assembly is spherical, τ_0 is 10.5 ns [19], and the motion of DPH bound in the assembly is severely restricted, the radius of the assembly was calculated to be 30 Å from the slope [17]. However, since DPH may be allowed to move in the assembly, the radius should be

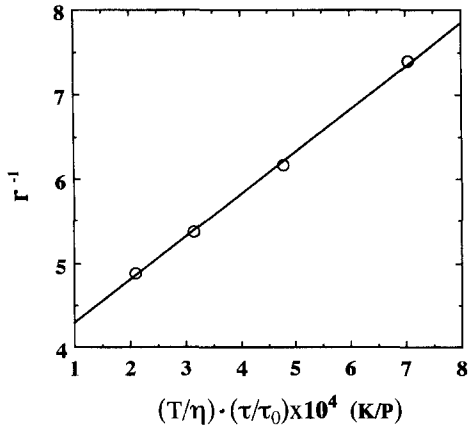

Fig. 2. Fluorescence depolarization of DPH in the presence of D9NT in a Tris buffer solution. The reciprocal of fluorescence anisotropy (r) at various temperatures is plotted against $(k_BT\tau)/(\eta\tau_0)$. τ/τ_0 is determined by $(I_p+2I_v)/(I_p+2I_v)$, where I_p and I_v represent fluorescence intensities of parallel and vertical orientation, respectively. I_p and I_v are those at 298 K.

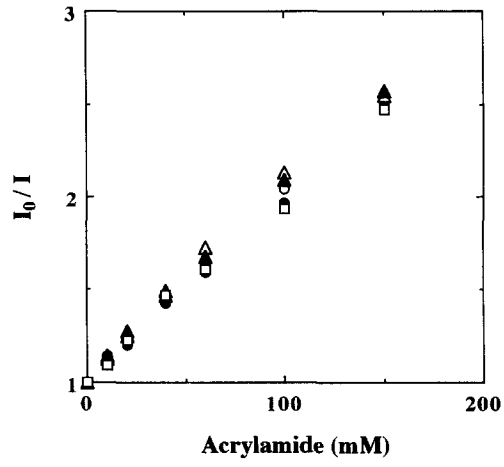

Fig. 4. The Stern–Volmer plot of fluorescence quenching with acrylamide of the neurotensin derivatives in the presence of DMPC liposome. D0NT (●), D3NT (△), D6NT (▲), D9NT (□), and neurotensin 8–13 fragment in a Tris buffer solution (○).

underestimated. The molecular length of D9NT should be in the range of 30–60 Å on the basis of molecular modeling, which depends on the conformation of the neurotensin fragment and the spacer chain. Consequently, it is reasonable to consider that D9NT takes a spherical micelle in water.

3.2. Interaction of neurotensin derivatives with liposome

The neurotensin derivatives were incubated in the presence of DMPC liposome overnight, and the distribution of the derivatives in the liposome was investigated. The elution profile of the dispersion of D3NT and DMPC liposome through a gel chromatography column showed a peak at the same position as DMPC liposome (Fig. 3), indicating that D3NT was bound to DMPC liposome.

The distribution rate of the derivatives to DMPC liposome was studied by measuring the amount of the peptide

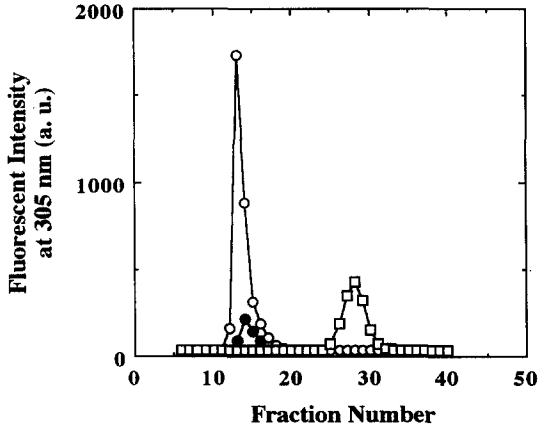

Fig. 3. The elution profile of DMPC liposome (●), D3NT immobilized on DMPC liposome (○), and neurotensin (□) through a Sephadex G-50 column. Experimental conditions are described in the text.

not included in liposome. When the neurotensin derivatives were incubated in the presence of DMPC liposome overnight and filtrated, the filtrate did not show fluorescence of Tyr residue of the neurotensin fragment. On the other hand, in the absence of DMPC liposome, the amounts of the derivatives found in the filtrate were 65% for D0NT, 91% for D3NT, 82% for D6NT, and 84% for D9NT. The relatively low value for D0NT is probably due to aggregation in a buffer solution, because D0NT is most hydrophobic among the derivatives. Therefore, it is concluded that nearly all of the neurotensin fragment derivatives are immobilized on DMPC liposome in water.

3.3. Location of neurotensin fragment in liposome

The location of the neurotensin message segment immobilized on DMPC liposome was determined by quenching of Tyr fluorescence of the neurotensin derivatives with a water-soluble quencher, acrylamide. All of the neurotensin derivatives were quenched with acrylamide in a similar manner to free neurotensin 8–13 fragment (Fig. 4). It is, therefore, considered that the neurotensin fragment of the derivatives is exposed to water phase with the dioctadecyl group anchoring to the hydrophobic core of DMPC liposome.

3.4. Receptor affinity of neurotensin derivatives immobilized on liposome

Receptor affinity of the neurotensin derivatives immobilized on liposome was investigated by the competitive binding assay using bovine brain homogenate. Fig. 5 shows the competitive inhibition of [^{3}H]neurotensin binding to the receptor with varying concentrations of peptide. The inhibition curve shifts to lower concentrations of D0NT upon immobilization on liposome, indicating that the re-

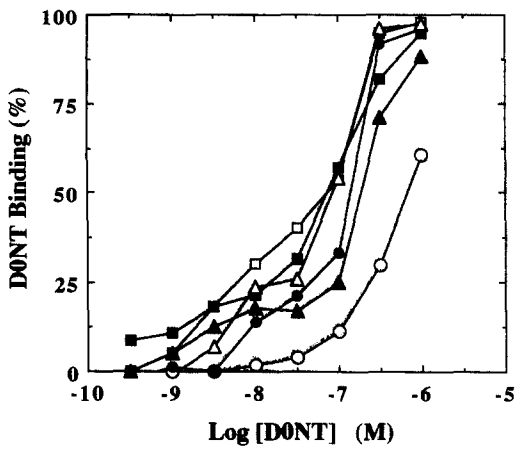

Fig. 5. Inhibition by D0NT of the [^{3}H]neurotensin binding to bovine brain homogenate in the presence of varying amounts of DMPC liposome. [DMPC]/[D0NT] = 0 (○), 50 (●), 100 (■), 200 (□), 500 (△), and 1000 (▲).

ceptor affinity of D0NT increases upon immobilization. The receptor affinity is dependent on the molar ratio of the lipid and the peptide. The highest affinity was obtained at the molar ratio of 200, where the receptor affinity of D0NT was 65-fold higher than that in the absence of liposome.

The IC_{50} values of D3NT with varying molar ratios of the lipid and the peptide are summarized in Table 1. The highest affinity was also obtained at the molar ratio of 200, and the affinity is similar to that of the neurotensin 8–13 fragment. The dependence of the affinity on the molar ratio may be explained by multisite interactions between the peptides immobilized on liposome and the receptors located in membrane. When the molar ratio is high, simultaneous binding should be difficult to occur. On the other hand, a high density of peptides on liposome should enable multisite interaction to occur easily, resulting in a high receptor affinity. When the density of the ligand in lipo-

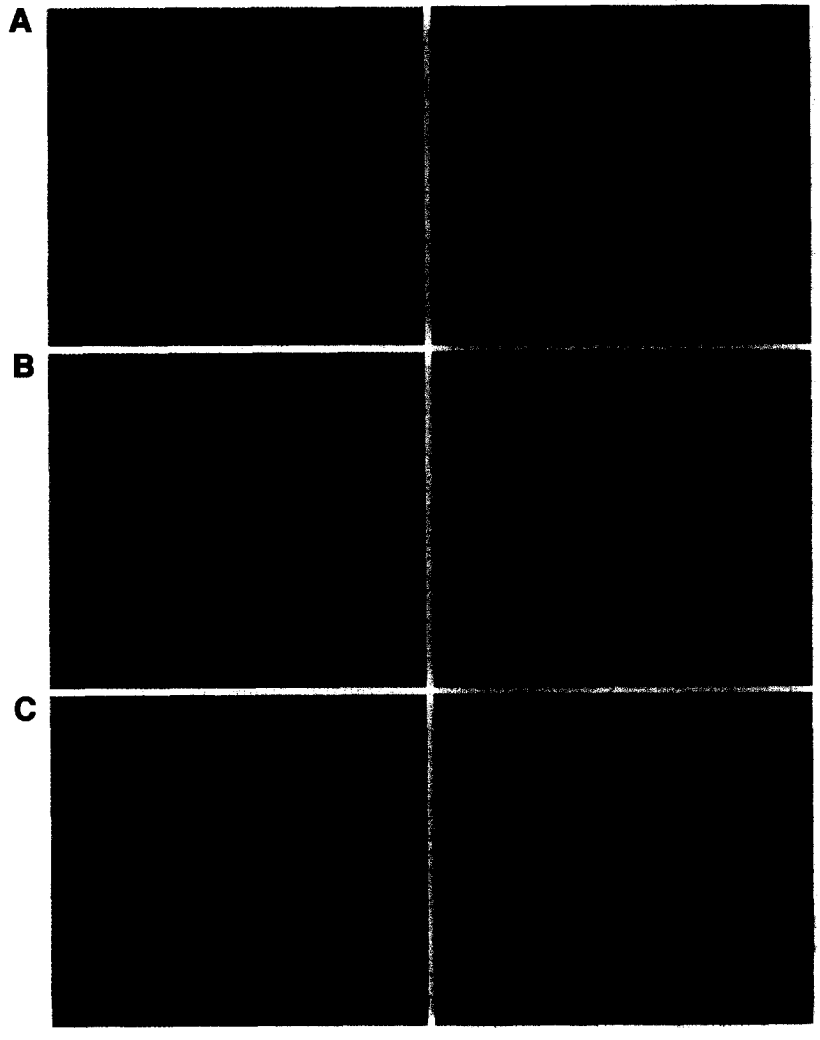

Fig. 6. Optical (left) and fluorescence micrographs (right) of NG108-15 cells incubated with (A) DMPC liposome containing Rho-DPPE, (B) neurotensin 8–13 fragment and DMPC liposome containing Rho-DPPE, (C) D9NT immobilized in DMPC liposome containing Rho-DPPE.

Table 1
Inhibition of [^{3}H]neurotensin binding to neurotensin receptor by D3NT with varying [DMPC]/[D3NT] at 20°C

[DMPC]/[D3NT] [a]	IC_{50} (nM)
0	36.4±3.6
50	18.4±3.6
100	13.6±3.1
200	10.0±1.4
500	15.5±3.7
1000	33.8±4.2
2500	29.6±5.4

[a] D3NT solution of $6 \cdot 10^{-5}$ M was incubated with DMPC liposome at 30°C overnight.

some is very high, some ligand molecules would not participate in the receptor binding. Consequently, the receptor affinity in terms of the ligand concentration may decrease, yielding the maximum value at the molar ratio of 200.

IC_{50} values of the neurotensin derivatives immobilized on liposome at the molar ratio of 200 are summarized in Table 2. All the peptides raise the receptor affinity upon immobilization on liposome, although the receptor affinity of neurotensin 8–13 fragment is independent of the presence of liposome. Notably, D9NT immobilized on liposome shows a trend of slightly higher receptor affinity than the neurotensin 8–13 fragment.

The receptor affinity changes with varying lengths of the spacer chain connecting the neurotensin fragment to the dioctadecyl group. The spacer chain is composed of -(Sar-Sar-Pro)$_n$- oligopeptide, which is hydrophilic and flexible. The receptor affinity of the neurotensin derivatives increases with elongation of the spacer chain either in free or in immobilized state. This observation may be explained in terms of a release of receptor binding from steric hindrance. With elongation of the hydrophilic spacer chain, the neurotensin fragment of the derivative may extend to aqueous phase which is sterically less crowded.

3.5. Observation of receptor binding by fluorescence microscopy

The binding of the neurotensin derivatives immobilized on liposome to receptors in NG108-15 cell was investigated by fluorescence microscopy. Liposome was labelled with Rho-DPPE. NG108-15 cell was not stained by liposome containing Rho-DPPE either in the absence or in the presence of neurotensin 8–13 fragment (Fig. 6 (A), (B)). However, NG108-15 cell became fluorescent when incubated with liposome containing Rho-DPPE and D9NT (Fig. 6 (C)). NG108-15 cell was made fluorescent by liposome containing Rho-DPPE in the presence of other neurotensin derivatives, too. It is, therefore, concluded that the neurotensin derivatives are bound by receptors as being tightly immobilized on liposome.

Table 2
Inhibition of [^{3}H]neurotensin binding to neurotensin receptor by neurotensin derivatives at 20°C

	IC_{50} (nM)	
	free	in liposome [a]
Neurotensin 1–13	4.5± 1.0	
Neurotensin 8–13	11.4± 3.4	12.9± 2.8
D0NT	615 ±42	70.0±10
D3NT	36.4± 3.6	10.0± 1.4
D6NT	46.6± 6.0	13.4± 2.7
D9NT	27.2± 4.1	7.0± 2

[a] [Neurotensin unit]/[DMPC] = 1:200.

3.6. Conclusion

The neurotensin derivatives having a dioctadecyl group at the N-terminal side are easily immobilized on DMPC liposome. The receptor affinity of the neurotensin derivatives increases upon immobilization on liposome. Especially, the neurotensin derivative with a long hydrophilic spacer chain is more familiar to the receptor than the neurotensin 8–13 fragment. Similar results were obtained by enkephalin derivatives having long alkyl chains which were immobilized on liposome [11]. It is, therefore, concluded that the immobilization of peptide ligands on liposome is an effective way to enhance the receptor affinity of the multivalent ligand system. The multivalent ligand system will simultaneously activate neighboring receptors existing in a localized area within the cell membrane, which may result in an enhanced chemical signaling, superpotency [7] or an altered signal transduction [9] due to receptor–receptor interactions [20]. The biological relevance of the multivalent ligand system is under investigation.

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