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MULTIVALENT LIGANDS FOR INDUCING RECEPTOR-RECEPTOR INTERACTIONS

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ABSTRACT

To obtain enchanced and/or unique cellular responses to peptide hormones by simultaneous activation of two different receptor systems, chimera peptide hormones in which enkephalin (EK) is connected through sarcosine oligomer (*n*) to the message segment of neurotensin [NT(8-13)] were synthesized. Interaction of these two receptor systems was studied by receptor-binding assay and determination of cAMP and cGMP level in cytosol of NG108-15 cells. It was found that EK-12-NT(8-13) induced specific cell response in terms of the second messenger level. The specific cell response was explained in terms of the enhancement of receptor–receptor interaction in cytosol due to activation of nearby occurring receptors of different kinds. It was also found that EK-12-NT(8-13) has a high affinity toward neurotensin receptor but a low affinity toward enkephalin receptor. To strengthen and prolong the activity of peptide hormones by simultaneous binding with two or more receptors in the target cell, enkephalin/lipid conjugates were immobilized on the surface of polymerized liposomes. It was found that multivalent ligands with a high receptor affinity were synthesized, which was sensibly influenced by the presence of a spacer chain connecting the enkephalin unit to the lipid part. It was found that the introduction of anionic charges to the polymerized liposomes strongly affected the receptor affinity of immobilized ligands.

INTRODUCTION

In the signal transmission of living cells, extracellular signal-transmission substances bind to their specific receptor molecules in cell membranes and transmit the biological signal to cytoplasm by activating intracellular signal-transmission systems intrinsic to the receptor molecules. Enhancement or suppression of interactions and feedback or feedforward interactions among the signal-transmission systems (receptor–receptor interactions) play important roles in the cell responses [1].

Specific cell responses such as intensification and prolongation of the activity might be brought about by specific and simultaneous activation of two signal-transmission systems of different kinds. According to this view, two kinds of peptide hormones working in the neurotransmission systems, enkephalin and neurotensin, were connected by a flexible spacer chain to produce a chimera peptide, and simultaneous activation of different kinds of receptors by the chimera peptide was investigated.

The structure of chimera peptide is shown in Fig. 1. The message fragment of enkephalin (EK) is connected through a hydrophilic and flexible spacer chain (*n*), which is sarcosine oligomer -(Sar)$_n$-, to the message fragment of neurotensin [NT(8-13)] to yield chimera peptides [EK-*n*-NT(8-13)], *n* being variable. The chimera peptides may activate specifically and simultaneously the adenylate cyclase system (the enkephalin system) and the phosphatidylinositol metabolic system (the neurotensin system).

The signal transmission of a biological system may also be strengthened by inducing interactions among nearby occurring receptor molecules by the action of multivalent ligand, in which a number of peptide hormones (ligand molecules) are connected to a polymeric carrier molecule such as protein and synthetic polymer [2, 3]. However, when a linear polymer was used for a carrier molecule, ligand molecules were buried in the carrier molecule to decrease the affinity toward receptor

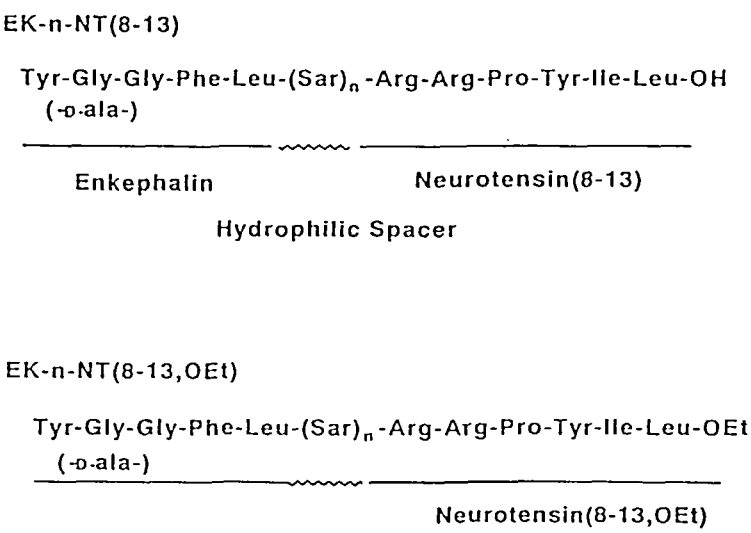

FIG. 1. The structure of enkephalin/neurotensin chimera peptides.

molecules [4]. To overcome the steric hindrance imposed by polymeric carrier molecules, polymerized liposomes were used for the carrier molecule on which many ligand molecules are immobilized, as shown in Fig. 2. Enkephalin/lipid conjugates, which are immobilized on the hydrophilic surface of the lipid bilayer membrane of the polymerized liposome, are easily accessible to receptor molecules on the biological membrane. Liposomes can be mechanically stabilized by polymerization. The immobilization of many ligand molecules onto the surface of the polymerized liposome can yield physiologically active multivalent ligand that inhibits down-regulation of receptor molecules. Multisite interaction of a polymerized liposome with many receptor molecules can be controlled by changing the distance between ligand molecules on the membrane surface [5], which is attained by changing the content of ligand molecules in the polymerized liposome.

The structures of enkephalin/lipid conjugates (ligand molecules) are shown in Fig. 3 together with a polymerizable lipid (MDL).To immobilize enkephalin onto polymerized liposome of MDL, the C-terminal of enkephalin was connected to dipalmitoyl phosphatidylethanolamine (DPPE) without a spacer chain (ENK/DPPE) or to dioleoyl phosphatidylethanolamine (DOPE) with a hydrophilic spacer chain (ENK-sp-DOPE).

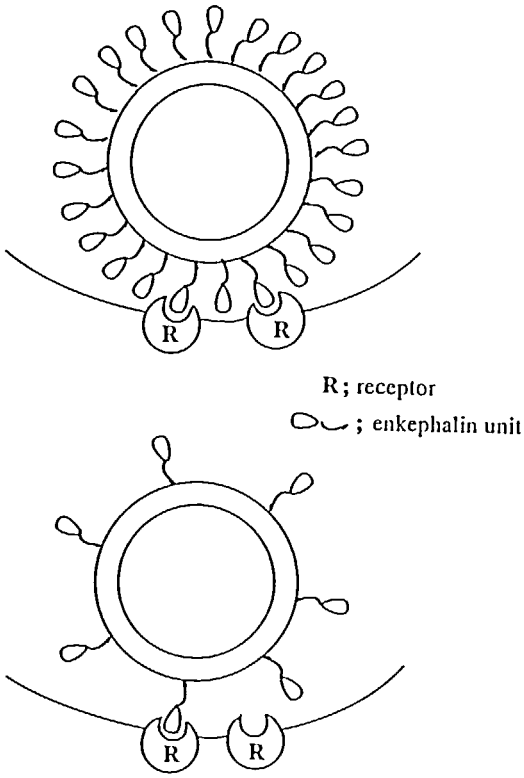

FIG. 2. A model for receptor binding of enkephalin/lipid conjugate immobilized on polymerized liposome.

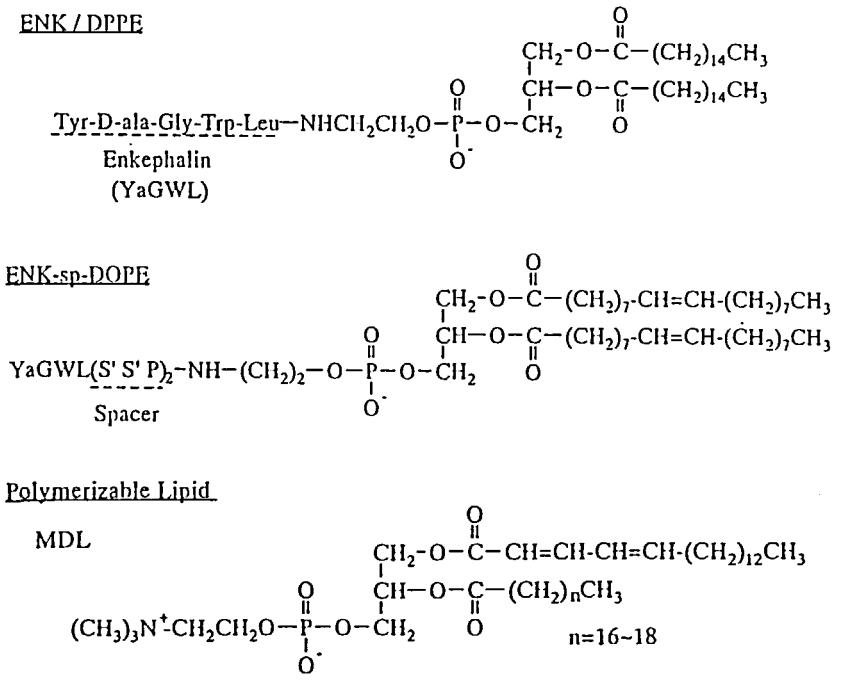

FIG. 3. The structure of enkephalin/lipid conjugates and polymerizable lipids.

In the present investigation the receptor–receptor interaction was investigated by using the chimera peptide ligand and the multivalent polymerized liposomes to create novel molecular systems having high receptor affinities.

EXPERIMENTAL

Synthesis

The enkephalin fragment in chimera peptides was synthesized by a conventional liquid-phase method, starting from Tyr-D-Ala-Gly-Phe which was synthesized by a solid-phase synthesis on an oxime resin [6] or from Tyr-Gly-Gly-Phe which was purchased from Bachem Co. The neurotensin fragment in chimera peptides was synthesized by a conventional liquid-phase method. The neurotensin fragment with blocking groups on side chains and C-terminal was used to initiate polymerization of Sar NCA. The chain length of the oligosarcosine fragment was evaluated by NMR spectroscopy. Subsequently, an elongation reaction by Boc-Tyr-Gly-Gly-Phe was carried out to synthesize chimera peptides. The final product was identified by NMR and amino acid analysis, and the purity was checked by HPLC.

In the synthesis of enkephalin/lipid conjugates, an enkephalin derivative, Tyr-D-Ala-Gly-Trp-Leu, was synthesized by a solid-phase synthesis on an oxime resin [6], in which the fourth Phe residue is replaced by Trp for fluorescence spectroscopy of peptides in lipid membrane. The hydrophilic spacer chain of hexapeptide of sarcosine and proline was synthesized by a conventional liquid-phase method [7].

The final product was obtained by the coupling reaction of the enkephalin unit, the spacer peptide, and phospholipid (DPPE or DOPE) and the subsequent deblocking of protective groups.

A polymerizable lipid, monodiene L-α-lecithin (MDL), and nonpolymerizable lipids, dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylglycerol (DPPG), phosphatidylserine (PS), cerebroside sulfate (CS) and *N*-dansyl dipalmitoyl phosphatidylethanolamine (DNS/DPPE), were commercially available.

Liposomes were prepared by ultrasonication of an aqueous lipid dispersion [8]. MDL liposome or MDL/DPPC (1/1 mol/mol) liposome was placed in a quartz cell and irradiated with a mercury-xenon lamp at 45°C. The polymerization of MDL was traced by decreasing absorption of the dienoyl group at 260 nm. The degree of polymerization of polymerized MDL was determined by GPC to be ~10. Polymerized liposomes were stained with phosphotungstic acid (2 wt%) and subjected to TEM observation. DSC of liposomes before and after the UV-light irradiation was measured at the heating rate of 1°C/min to determine the gel–liquid crystal phase-transition temperature.

Determination of Second Messenger Concentration

The concentration of cAMP and cGMP in the NG108-15 cell was determined by the radioimmunoassay method using the Amersham assay system.

Determination of Receptor Affinity

The receptor affinity of a ligand was determined by the inhibition of radioisotope-labeled ligand binding to the receptor [9]. The NG108-15 cells and the bovine brain homogenates were prepared for the biological membrane containing receptor molecules [^{3}H]DPDPE, [^{3}H]DAGO, and [^{3}H] or [^{125}I]neurotensin were used as radioisotope-labeled ligands specific to δ- and μ-enkephalin receptor and neurotensin receptor, respectively.

Spectroscopy

The interaction of enkephalin/lipid conjugate with liposome was investigated by fluorescence spectroscopy. Uptake of enkephalin/lipid conjugate by liposomes was investigated by the changes of tryptophane fluorescence. The location of the enkephalin/lipid conjugate in liposomes was investigated by the Stern–Volmer plot of fluorescence quenching with acrylamide.

The distribution state of the enkephalin/lipid conjugate in liposomes was investigated by an excitation energy transfer from ENK-sp-DOPE to DNS/DPPE which are contained in the liposome.

SIMULTANEOUS ACTIVATION OF RECEPTORS OF DIFFERENT KINDS BY CHIMERA PEPTIDE HORMONES

Intersystem Interaction

The occurrence of intersystem interactions between the adenylate cyclase suppression system (the enkephalin system) and the phosphatidylinositol metabolic system (the neurotensin system) has been made clear [10]. The intersystem interac-

tion is schematically shown in Fig. 4. Enkephalin suppresses the action of adenylate cyclase to decrease the intracellular concentration of cAMP which is a second messenger of the biological signal. Since cAMP suppresses the action of PLC, the action of enkephalin results in the activation of PLC and, in turn, the amplification of signal transmission through the neurotensin system. Other types of interactions between signal-transmission systems with the intervention of cGMP or Ca^{2+} might be possible.

Intracellular Concentration of cAMP

Various peptide ligands were added to NG108-15 cells, and the change of intracellular concentration of cAMP was investigated. As shown in Fig. 5, the concentration of cAMP was decreased by the addition of EK (0.1 μM) or NT(1-13) (0.1 μM). The simultaneous addition of EK and NT(1-13) gave nearly the same results as when EK alone was added, showing the absence of significant interactions between the two signal-transmission systems in terms of cAMP concentration.

On the other hand, the decrease of cAMP concentration was not observed when a chimera peptide EK-12-NT(8-13) was added. The reason for the specific behavior of the chimera peptide could be considered as follows. The bivalent ligand consisting of two monovalent ligands has decreased receptor affinity or became antagonist against each receptor. Alternatively, the chimera peptide crosslinks receptor molecules of different kinds to activate nearby occurring receptors, resulting in promotion of intersystem interactions.

Intracellular Concentration of cGMP

Various peptide ligands were added to NG108-15 cells, and the change of intracellular concentration of cGMP was investigated. As shown in Fig. 6, the concentration of cGMP was increased by the addition of NT(1-13) (0.1 μM), al-

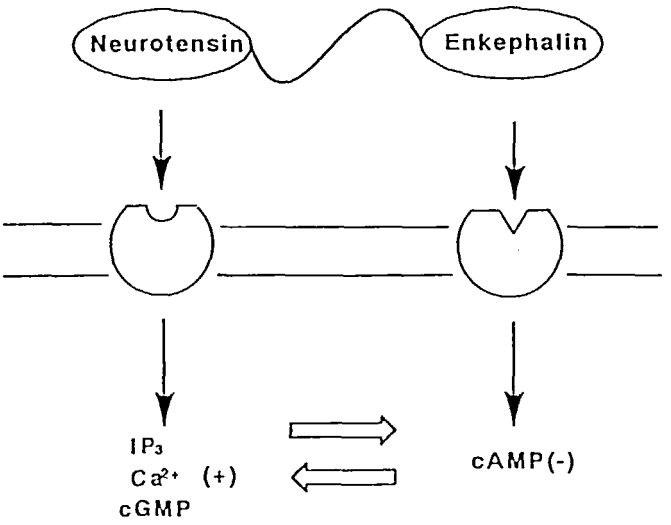

FIG. 4. Receptor–receptor interactions in the second messenger level.

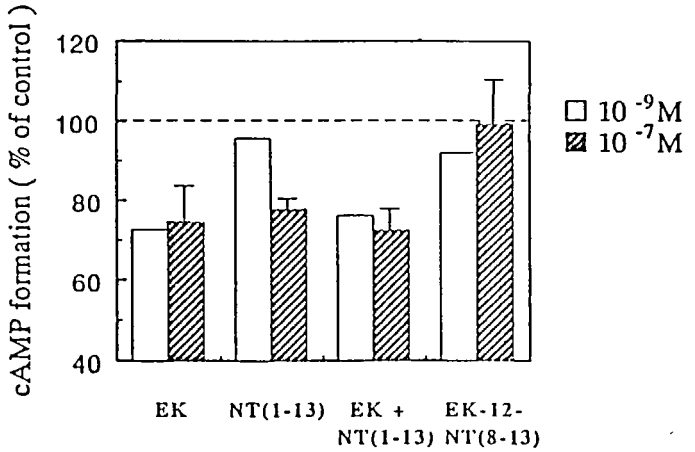

FIG. 5. cAMP formation in intact NG108-15 cell by various ligands.

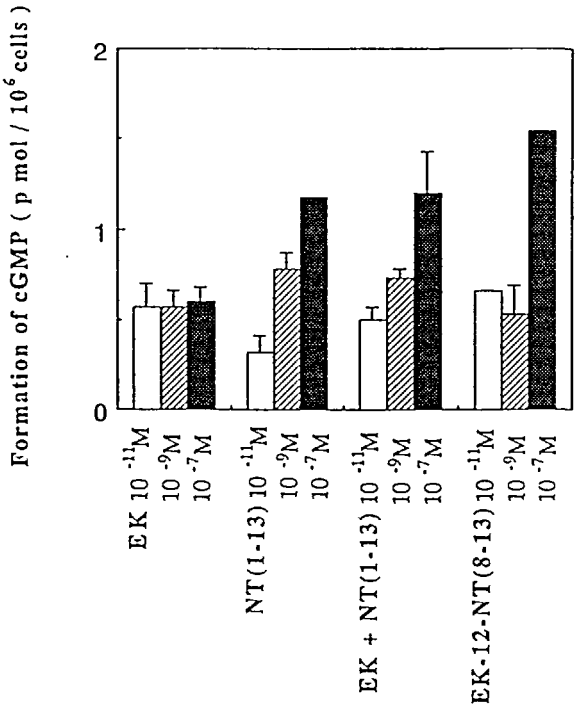

FIG. 6. cGMP formation in intact NG108-15 cell by various ligands.

though the addition of EK (0.1 μM) did not influence the cGMP concentration. The simultaneous addition of EK and NT(1-13) gave nearly the same results as when NT(1-13) alone was added, showing the absence of significant interactions between the two signal-transmission systems in terms of cGMP concentration. On the other hand, the addition of the chimera peptide induced a larger increase of cGMP concentration than the addition of NT(1-13) alone. The action mechanism of the chimera peptide could be explained in terms of crosslinking receptor molecules of different kinds to activate nearby occurring receptors, resulting in promotion of intersystem interactions.

Receptor Affinity of Enkephalin Fragment

Using bovine brain homogenate, the affinities of EK and a chimera peptide EK-12-NT(8-10, OMe) toward δ- and μ-receptors were determined. The receptor affinity is shown in Table 1 as the concentration of peptide ligand necessary to inhibit 50% of radioisotope-labeled ligand binding (IC_{50}). It is known that NT(8-10, OMe) is a neurotensin fragment which cannot bind to a neurotensin receptor by itself. It is found in Table 1 that the μ-receptor affinity of EK-12-NT(8-10, OMe) is nearly the same as that of EK. This result implies that elongation of the C-terminal of EK by a hydrophilic peptide segment does not affect the receptor binding of EK. The slight decrease of δ-receptor affinity of EK-12-NT(8-10, OMe) could be due to conversion of the carboxyl end group of EK to the amide bond in the chimera peptide.

Receptor Affinity of Neurotensin Fragment

Using bovine brain homogenate, the affinities toward neurotensin receptor of NT(1-13), NT(8-13), and NT(8-13, OEt) were determined. It is found in Table 2 that NT(8-13) has a higher receptor affinity than NT(1-13), whereas NT(8-13, OEt) has a lower receptor affinity than NT(1-13). These results indicate that the effective

TABLE 1. Receptor Affinities of Enkephalin and Enkephalin Derivatives

	IC_{50} (nM)		
Ligand	δ-Receptor	μ-Receptor	NT-Receptor
EK	3.4 ($\pm$ 1.7)	6.9 ($\pm$ 1.6)	>1000
EK-NH_2	17.0	—	—
EK-12-NT(8-10, OMe)	11.1 ($\pm$ 3.7)	6.1 ($\pm$ 0.1)	—

TABLE 2. Receptor Affinities of Neurotensin Fragments

Ligand	IC_{50} (nM)		
	δ-Receptor	μ-Receptor	NT-Receptor
NT(1-13)	>1000	>1000	9.2 (± 2.1)
NT(8-13)	–	–	6.3 (± 0.7)
NT(8-13, OEt)	–	–	24

binding of neurotensin to the receptor requires the presence of the 8th to 13th residues in the C-terminal region and that the C-terminal be a free carboxyl group.

Receptor Affinity of Chimera Peptide

Using bovine brain homogenate, the affinities toward a neurotensin receptor of EK-*n*-NT(8-13) chimera peptides having different lengths of oligosarcosine spacer chain (*n*) were determined, and the results are shown in Table 3. It is found in Table 3 that the neurotensin receptor affinity of chimera peptide varies with changing lengths of spacer chain. In the case of $n = 12$, the receptor affinity reached the maximum. EK-12-NT(8-13) has a higher affinity toward a neurotensin receptor than NT(1-13). The receptor affinity decreased in the presence of an excess amount of EK.

These experimental results suggest crosslinking of receptor molecules of different kinds by the chimera peptides. However, a different explanation exists: that the chimera peptides crosslink a neurotensin receptor and its subsite which is located near the neurotensin receptor for binding an enkephalin fragment. In fact, a chimera peptide without a spacer chain, EK-NT(8-13), which is considered to be ineffective in crosslinking receptor molecules of different kinds, showed a high affinity toward a neurotensin receptor. A high receptor affinity was not observed in the presence of an excess amount of EK. These observations support the above-men-

TABLE 3. Neurotensin Receptor Affinities of Chimera Peptides

Ligand	IC_{50} (nM)
NT(1-13)	9.2 (± 2.1)
EK-NT(8-13)	8.3 (± 2.8)
+ EK (1×10^{-4} M)	47
EK-8-NT(8-13)	7.9 (± 2.8)
+ EK (1×10^{-4} M)	37
EK-12-NT(8-13)	4.5 (± 1.2)
+ EK (1×10^{-4} M)	19
EK-24-NT(8-13)	19 (± 2.6)
+ EK (1×10^{-4} M)	41

tioned explanation that the chimera peptides crosslink a neurotensin receptor and its subsite. The mechanism of receptor binding by chimera peptides is illustrated in Fig. 7.

Using bovine brain homogenate, the affinities toward the μ-receptor of EK-*n*-NT(8-13) chimera peptides having different lengths of oligosarcosine spacer chain (*n*) were determined, and the results are shown in Table 4. It is found in Table 4 that the μ-receptor affinity decreased in the presence of an excess amount of NT in the case of $n = 8$, 12, and 24. These results suggest that these ligands crosslink receptor molecules of different kinds. However, the receptor affinities of the chimera peptides were much lower than those of EK.

OPIOID RECEPTOR AFFINITY OF ENKEPHALIN/LIPID CONJUGATE IMMOBILIZED ON POLYMERIZED LIPOSOME

Synthesis and Properties of Polymerized Liposome

A polymerizable lipid (MDL) and one of the usual nonpolymerizable lipids were mixed to the compositions shown in Table 5, and the liposomes of mixed lipids

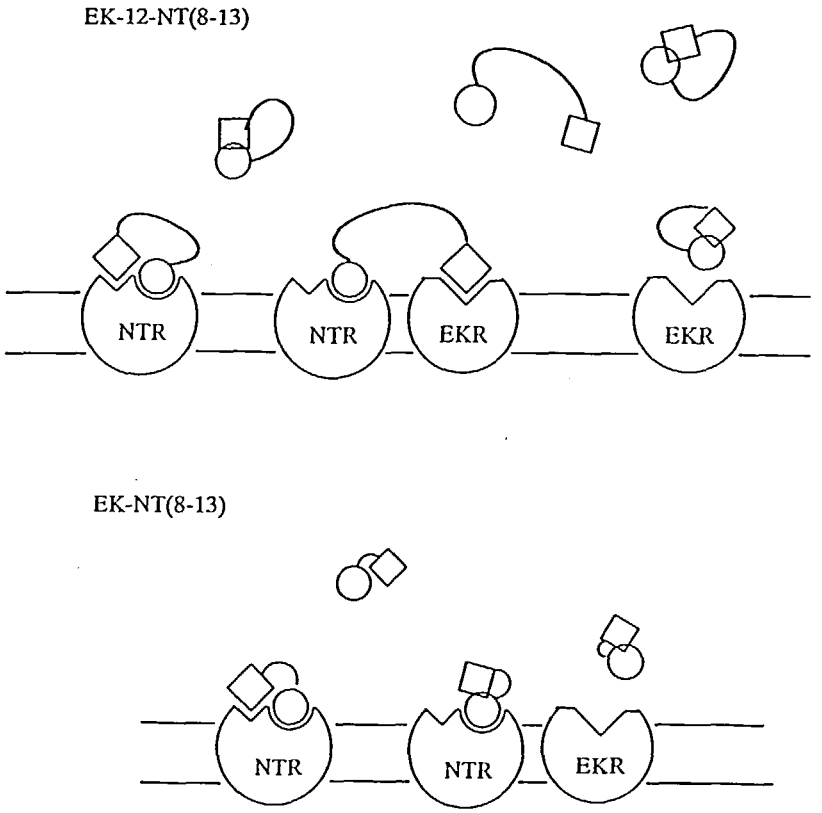

FIG. 7. The mechanism of receptor binding by chimera peptides: EKR, enkephalin receptor; NTR, neurotensin receptor.

TABLE 4. μ-Receptor Affinities of Chimera Peptides

Ligand	IC_{50} (nM)
EK	6.9 (± 1.6)
EK-NT(8-13)	530 (± 26)
+ NT (1×10^{-5} M)	400
EK-8-NT(8-13)	190 (± 36)
+ NT (1×10^{-5} M)	250
EK-12-NT(8-13)	270 (± 19)
+ NT (1×10^{-5} M)	490
EK-24-NT(8-13)	140 (± 8)
+ NT (1×10^{-5} M)	190

were polymerized with UV-light irradiation to yield polymerized liposomes having diameters of 30–40 nm.

In the DSC analysis the polymerized PL1 showed an endotherm due to gel-liquid crystal phase transition at 37.3°C. The polymerized PL4 showed three endothermic peaks at 33.1, 38.3, and 41.6°C. The endotherm is ascribed to the phase transitions occurring in the MDL oligomer/DPPC mixed domain, MDL oligomer domain, an DPPC domain, respectively.

Opioid Receptor Affinity of Enkephalin/Lipid Conjugate

Enkephalin/lipid conjugates ENK/DPPE and ENK-sp-DOPE were introduced to polymerized liposome PL1 in various molar ratios, and the opioid receptor affinity of the enkephalin lipid immobilized on the polymerized liposome was determined by inhibition of radioisotope-labeled ligand binding to bovine brain homogenate. The IC_{50} values are shown in Table 6 where the numerical values shown in parentheses represent the initial molar ratio of mixing enkephalin/lipid conjugate and polymerized liposome.

TABLE 5. Composition of Liposomes

Notation	Liposome composition
PL1	MDL 100%
PL4	[MDL]/[DPPC] = 50/50
PLG	[MDL]/[DPPG] = 88/12
PLS	[MDL]/[PS] = 88/12
PLC12	[MDL]/[CS] = 88/12
PLC25	[MDL]/[CS] = 75/25
C100	CS 100%

TABLE 6. Inhibition of [^{3}H]DPDPE and [^{3}H]DAGO Binding to Bovine Brain Homogenate by Neutral Enkephalin/Lipid Conjugates at 20°C

	IC_{50} (nM)	
	[^{3}H]DPDPE (δ)	[^{3}H]DAGO (μ)
ENK-sp-PL1(1/75)	68 ± 2.0	30 ± 3.3
ENK-sp-PL1(1/340)	15 ± 2.6	26 ± 2.6
ENK-sp-PL1(1/3000)	47 ± 12	42 ± 5.3
ENK-sp-PL1(1/6000)	43 ± 12	59 ± 21
ENK-sp-PL1(1/9000)	120 ± 37	180 ± 55
ENK/PL1(1/75)	270 ± 54	330 ± 54
ENK-sp-DOPE	190	120
ENK/DPPE	180	
YGGFL	4.1 ± 0.4	15 ± 0.8
Enkephalin/dextran	110 ± 14	820 ± 76

It is found in Table 6 that the receptor affinity of enkephalin/lipid conjugate with a spacer chain is higher than that without a spacer chain. The enkephalin unit in the former conjugate should be located at a distance from the membrane surface due to the flexible and hydrophilic spacer chain, which diminishes the steric effects of liposomes on receptor binding. The lower receptor affinity of free ENK-sp-DOPE than of that immobilized on polymerized liposomes may be explained in terms of an aggregation of ENK-sp-DOPE in aqueous solution which inhibits receptor binding sterically. Furthermore, variation of the ENK-sp-DOPE/PL1 mixing ratio influenced receptor affinity, implying the occurrence of multisite binding between the ligands immobilized on the polymerized liposome and the receptors.

However, enkephalin/lipid conjugates immobilized on the membrane surface showed lower affinities toward μ- and δ-receptors than did the free enkephalin. The lower receptor affinity of the former system is ascribed to nonspecific binding of polymerized liposomes to the bovine brain homogenate membrane which decreases the effective binding of the enkephalin unit. This nonspecific binding is possibly due to hydrophobic interactions and should be overcome by increasing the hydrophilicity of the polymerized liposome. According to this consideration, MDL was mixed with various anionic lipids (DPPG, PS, or CS) in the molar ratios indicated in Table 5, and the mixed-lipid liposomes were polymerized. Enkephalin/lipid conjugate ENK-sp-DOPE was introduced to the polymerized liposomes. The μ- and δ-receptor affinities of ENK-sp-DOPE immobilized on the anionic polymerized liposomes were determined by inhibition of radioisotope-labeled ligand binding to bovine brain homogenate, and they are shown in Table 7 in terms of IC_{50}.

It is found in Table 7 that the δ-receptor affinity of ENK-sp-DOPE increases with an increasing content of anionic lipid in the polymerized liposome, while the μ-receptor affinity increases with a decreasing content of anionic lipid. The opposite influences of anionic lipid on μ- and δ-receptor affinities may be related to the state of the enkephalin/lipid conjugate in polymerized liposomes.

TABLE 7. Inhibition of [^{3}H]DPDPE and [^{3}H]DAGO Binding to Bovine Brain Homogenate by Anionic Enkephalin/Lipid Conjugates at 20°C

	IC_{50} (nM)	
	[^{3}H]DPDPE (δ)	[^{3}H]DAGO (μ)
ENK-sp-PL1	15 ± 2.6	26 ± 2.6
ENK-sp-PL4	48 ± 2.7	44 ± 7.2
ENK-sp-PLG	20 ± 2.2	25 ± 4.7
ENK-sp-PLS	19 ± 9.2	20 ± 0.3
ENK-sp-PLC12	19 ± 2.5	17 ± 1.2
ENK-sp-PLC25	7.4 ± 1.8	25 ± 1.5
ENK-sp-C100	22 ± 1.8	36 ± 3.2
YGGFL	4.1 ± 0.4	15 ± 0.8

Distribution of Enkephalin/Lipid Conjugate in Polymerized Liposome

The distribution of the enkephalin unit in the polymerized liposomes PL1, PLC12, and PLC25 was examined by fluorescence spectroscopy of the tryptophane residue (340 nm). It was found that the distribution coefficient of ENK-sp-DOPE was not influenced by the nature of the polymerized liposomes, while the fluorescence quenching with the double bond of polymerized liposome increased with increasing content of anionic lipid. It was therefore concluded that polymerized liposomes containing a higher amount of anionic lipid takes up ENK-sp-DOPE nearer to the CS double bond.

Location of Enkephalin/Lipid Conjugate in Polymerized Liposome

The location of the enkephalin unit in the polymerized liposomes PL1, PLC12, and PLC25 was investigated by fluorescence quenching experiment using a water-soluble quencher, acrylamide. The Stern–Volmer plot of the quenching experiment showed that the quenching efficiency increased with increasing CS content in the polymerized liposomes. This experimental result together with the results of the distribution experiment indicate that the enkephalin unit of the conjugate is located closer to the member surface with increasing anionic lipid content due to electrostatic interactions with the sulfate group of CS, which makes the membrane surface increasingly hydrophilic.

Dispersion State of Enkephalin/Lipid Conjugate in Polymerized Liposome

The dispersion state of ENK-sp-DOPE in the polymerized liposome PL1 containing DNS/DPPE was estimated from the energy-transfer efficiency from the tryptophane residue to the dansyl group. After correcting for the quantum yield of Trp residue, the energy-transfer efficiency in PLC12 (50.6%) was higher than those

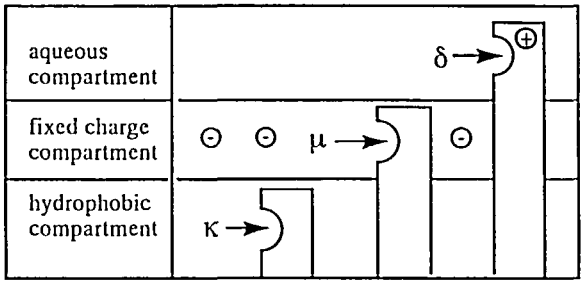

FIG. 8. The membrane compartment concept proposed by Schwyzer for the access of opioid ligands to the κ-, μ-, and δ-receptors.

in PL1 (37.2%) and PLC25 (35.2%). It is therefore concluded that the interligand distance is shortest in anionic polymerized liposome PLC12.

Opioid Receptor Affinity of Enkephalin/Lipid Conjugate immobilized on Polymerized Liposome

The affinity toward opioid receptors of the enkephalin/lipid conjugate immobilized on the polymerized liposomes is discussed on the basis of the results of fluorescence spectroscopy.

The binding sites of the opioid receptor are located in specific regions of the lipid membrane as shown in Fig. 8. Since the binding site of δ-receptor exists in an aqueous region,the ligand binding to δ-receptor is strongly influenced by the hydrophilicity of the ligand and carrier molecule. In the present investigation, δ-receptor affinity increased with increasing hydrophilicity of the polymerized liposomes.

On the other hand, the binding site of the μ-receptor exists in a charged region of the lipid membrane, and electrostatic interactions play an important role in binding to the μ-receptor. Therefore, the μ-receptor affinity of ENK-sp-DOPE immobilized on PLC25, which carries a high amount of anionic charges, decreased due to electrostatic repulsion with electric charges on the bovine brain homogenate membrane. Furthermore, the higher affinity toward the μ-receptor of ENK-sp-DOPE immobilized on PLC12, in which the distance between the ligand groups is shortest, suggests that the distance between the binding sites of adjacent μ-receptors is short.

Several possibilities have been proposed that μ- and δ-receptors form a complex [11] and that a μ-receptor molecule possess more than two binding sites [12, 13]. Taking these possibilities into consideration, the results of the present investigation of polymerized liposomes carrying an enkephalin/lipid conjugate can be explained on the basis of receptor–receptor interaction.

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