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# Interaction of a cyclic peptide, Ro09-0198, with phosphatidylethanolamine in liposomal membranes

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Key words: Cyclic peptide; Ro09-0198; Phosphatidylethanolamine; Liposome permeability; (*Streptoverticillium*)

**Ro09-0198 is a cyclic peptide isolated from *Streptoverticillium griseoverticillatum*. This peptide caused permeability increase and aggregation of liposomes containing phosphatidylethanolamine. Liposomes containing phosphatidylserine, phosphatidylinositol or cardiolipin instead of phosphatidylethanolamine were, however, not appreciably reactive with the peptide. Among the structural analogs of phosphatidylethanolamine, dialkylphosphatidylethanolamine and 1-acylglycerophosphoethanolamine incorporated into liposomes could interact with Ro09-0198 to cause a permeability increase, whereas liposomes consisting of alkylphosphoethanolamine or phosphatidyl-*N*-monomethylethanolamine were insensitive to the peptide. These findings indicate that a glycerol backbone and a primary amino group of phosphatidylethanolamine are necessary for interaction with Ro09-0198 to cause membrane damage. Ro09-0198 induced a selective permeability change on liposomes. Glucose and umbelliferyl phosphate were effluxed significantly, but sucrose was only slightly permeable and inulin could not be released. Consequently, the permeability increase induced by Ro09-0198 is rather specific to molecules smaller than sucrose. Line broadening of electron spin resonance signals of spin-labeled phosphatidylethanolamine was observed upon treatment of liposomes with Ro09-0198. It was suggested from these results that Ro09-0198 can alter the physical organization of phosphatidylethanolamine in membranes, thus providing a basis for changes in membrane permeability.**

## Introduction

Ro09-0198 is a cyclic peptide isolated from culture filtrates of *Streptoverticillium griseoverticillatum* [1]. It consists of 15 amino acids, including unusual amino acids such as lanthionine, β-methyllanthionine, lysinoalanine, β-hydroxyaspartic acid and D-phenylalanine, and has a high amount of intramolecular cross-linking [2].

This peptide has an antimicrobial activity against Gram-positive bacteria [1] and induces lysis of erythrocytes of various animal species [3]. As reported previously [3], the peptide recognizes precisely the chemical structure of phosphatidylethanolamine and can bind to it. Other phospholipids present in erythrocytes in nature cannot interact with the peptide. Several biological substances are known to interact specifically with some membrane lipid constituent causing a change of membrane functions, such as polyene antibiotics to sterols [4,5], cholera toxin to gangliosides [6,7]. There has been, however, little information about the compounds which are reactive specifi-

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cally to the particular phospholipids except for neomycin which can interact with phosphatidylinositol 4,5-bisphosphate [8]. Duramycin is a structurally related compound to Ro09-0198 [12]. It was shown that duramycin can be also active against biological membranes [9–12]. It was also reported that duramycin interacted with phosphatidylethanolamine as well as glyceroglycolipid, causing agglutination of the lipid suspensions [12]. Very little, however, is known about mechanisms for the interaction of these peptides with phosphatidylethanolamine on biological membranes leading to the membrane damage.

In the present work, we found that Ro09-0198 causes marker release from liposomes containing phosphatidylethanolamine. The mechanism of the increase in permeability of liposomes caused by this peptide has been studied.

## Materials and Methods

*Materials.* Ro09-0198 was isolated from culture broths of *S. griseoverticillatum* as described previously [1] and further purified on a preparative HPLC C-18 column. Egg-yolk phosphatidylcholine and egg-yolk phosphatidylethanolamine were prepared by chromatography on neutral aluminium oxide and silicic acid columns. Cardiolipin from bovine heart was prepared by the method of Faure and Maréchel [13]. Bovine brain phosphatidylserine was isolated as described previously [14]. Yeast phosphatidylinositol was prepared by the method of Trevelyan [15]. 1-Acylglycerophosphoethanolamine was obtained from egg-yolk phosphatidylethanolamine by treatment with phospholipase $A_2$ from snake venom (*Naja naja*) and purified by chromatography on a silicic acid column Dialkylphosphatidylethanolamine and alkylphosphoethanolamine were prepared by means of a transphosphatidylation reaction catalyzed by phospholipase D as described previously [16]. Dicetylphosphate, cholesterol, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, phosphatidyl-*N*-monomethylethanolamine and 4-methylumbelliferyl phosphate were purchased from Sigma, St. Louis, MO, U.S.A. D-[6-$^3$H]Glucose (22.5 Ci/mol), D-[U-$^{14}$C]sucrose (1.24 Ci/mol) and [$^3$H]inulin (37.8 Ci/mol) were purchased from New England Nuclear, Boston, MA, U.S.A. A stearic acid derivative (12-(*N*-oxy-4′,4′-dimethyloxazolidine)stearate), which has a nitroxide-containing ring in the C-12 position, was synthesized by the method of Waggoner et al. [17]. Spin-labeled phosphatidylcholine (12SL-phosphatidylcholine) was synthesized from egg yolk phosphatidylcholine according to the method of Hubbell and McConnell [18]. Spin-labeled phosphatidylethanolamine (12SL-phosphatidylethanolamine) was prepared from 12SL-phosphatidylcholine by transphosphatidylation reaction of phospholipase D and purified by chromatography on a silicic acid column.

*Buffer.* Tris-buffered saline (10 mM Tris-HCl (pH 7.4)/150 mM NaCl) was used throughout the present study except when otherwise noted.

*Erythrocytes.* The human erythrocytes used were from freshly drawn, heparinized blood of healthy donors. The blood was centrifuged at $300 \times g$ for 5 min at room temperature and the plasma and buffy coat were carefully discarded by aspiration. The precipitated cells were then washed three times with 10 vol. of ice-cold medium used in the experiments and used within 48 h.

*Preparation of liposomes.* Reverse-phase evaporation vesicles were prepared as described previously [19]. Untrapped [$^3$H]glucose, [$^{14}$C]sucrose, [$^3$H]inulin and 4-methylumbelliferyl phosphate were removed by dialyzing the preparations at room temperature against Tris-buffered saline for more than 2 h.

*Measurement of liposomal permeability.* Umbelliferyl phosphate release from liposomes was assayed by enzymatic hydrolysis of the compound with alkaline phosphatase to phosphate and the fluorophore, 4-methylumbelliferone, by the method of Six et al. [20]. Liposomes (10 nmol as phospholipids) were incubated with various amounts of Ro09-0198 in Tris-buffered saline (1 ml) containing 0.1 U of alkaline phosphatase from *E. coli* (Sigma). The amount of umbelliferone genarated was determined fluorometrically with a spectrofluorophotometer (Simadzu Seisakusho RF-501) using excitation and emission wavelengths of 340 and 448 nm, respectively. The total amount of umbelliferyl phosphate trapped in liposomes was determined by adding 100 $\mu$l of 10% Triton X-100.

Release of [$^3$H]glucose, [$^{14}$C]sucrose and [$^3$H]inulin from liposome were determined using

an ultrafiltration cell as described previously [21]. In brief, liposomes (10 μM as phospholipids) containing traces of radioactive markers were incubated with various amounts of Ro09-0198 in 0.3 M glucose solution at 37°C for 30 min. The reaction mixtures were poured into the ultrafiltration cell (Amicon Model 12, Amicon Corp.) equipped with a Millipore membrane filter (type PH, pore size 0.3 μm), and the radioactivity in aliquots (1 ml) of the filtrate was counted.

*Measurement of agglutination of liposomes.* Liposomes (10 μM as phospholipids) were incubated with various amounts of Ro09-0198 at 37°C for 30 min. Agglutination of liposomes was determined by measuring the change in absorbance at 340 nm as described previously [22].

*Inhibition of Ro09-0198-induced hemolysis by liposomes.* 0.86 nmol of Ro09-0198 was preincubated with various amounts of liposomes in 600 μl of Tris-buffered saline. Then 400 μl of human erythrocyte suspension ($2.5 \cdot 10^7$ cells/ml) labeled with $^{51}Cr$ was added to the reaction mixture and incubated at 37°C for 30 min. The hemolysis was determined as described previously [3].

*ESR measurements.* Liposomes (1 mM as phospholipids) consisting of egg-yolk phosphatidylethanolamine/12SL-phosphatidylethanolamine or 12SL-phosphatidylcholine/egg-yolk phosphatidylcholine/dicetylphosphate/cholesterol (molar ratio, 5 : 0.5 : 5 : 1 : 10) were incubated with Ro09-0198 at 37°C for 30 min. The reaction mixtures were transferred to sealed capillary tubes and the electron spin resonance spectra were measured at 37°C with a JES-PE-1X, X band, 100 kHz field modulator (JEOL, Tokyo, Japan).

## Results

*Permeability increase and aggregation of liposomes containing phosphatidylethanolamine by Ro09-0198*

When liposomes composed of egg-yolk phosphatidylcholine, egg-yolk phosphatidylethanolamine, dicetyl phosphate and cholesterol (0.75/0.25/0.1/1.0, molar ratio) were incubated with Ro09-0198 for 30 min at 37°C, release of the entrapped marker, umbelliferyl phosphate from these liposomes was observed (Fig. 1A). Permeability increase was dependent on the concentration of the peptide. About 2.5 μM of Ro09-0198 was required for 50% release of umbelliferyl phosphate entrapped in liposomes. Ro09-0198 also induced the aggregation of the liposomes with phosphatidylethanolamine, since the turbidity in suspensions of liposomes containing phosphatidylethanolamine was increased with increasing concentration of the peptide (Fig. 1B). These findings indicate that the permeability increase of the

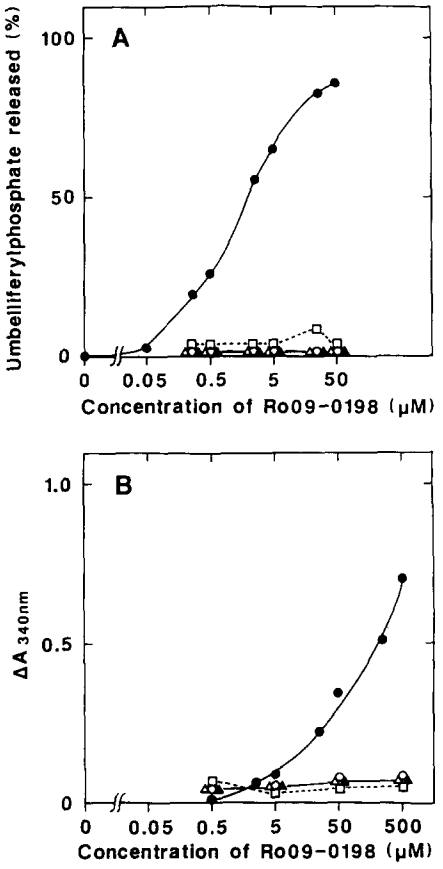


Fig. 1. Effect of Ro09-1098 on liposomes composed of various kinds of phospholipid. Liposomes were composed of egg-yolk phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio, 0.75 : 0.1 : 1.0) without (○) or with egg-yolk phosphatidylethanolamine (●), phosphatidylserine (□), phosphatidylinositol (▲) or cardiolipin (△) at molar ratio of 0.25 to phosphatidylcholine. Permeability change (A). Liposomes (10 μM as phospholipids) containing umbelliferyl phosphate in their aqueous compartments were incubated with various concentrations of Ro09-0198 at 37°C for 30 min and then the release of umbelliferyl phosphate from liposomes was measured. Turbidity change (B). Liposomes (10 μM as phospholipids) were incubated with various concentrations of Ro09-0198 at 37°C for 30 min. The change in optical absorbance of the reaction mixture was measured at 340 nm.

liposomes caused by the peptide is much more sensitive than the liposome agglutination. Liposomes consisted of phosphatidylserine, phosphatidylinositol or cardiolipin instead of phosphatidylethanolamine were not appreciably reactive with the peptide, indicating that Ro09-0198 interacts specifically with phosphatidylethanolamine on liposomal membranes and causes changes in the physical organization of membranes. The time-course of umbelliferyl phosphate release from liposomes containing phosphatidylethanolamine is shown in Fig. 2A. The release reached to the maximum after 10 min with 5 μM of Ro09-0198. The rate of umbelliferyl phosphate release by the peptide was independent of temperature, since the release at 0 °C was the same as that observed at 37 °C. The sensitivity of liposomes to the peptide increased with increase of phosphatidylethanolamine content (Fig. 2B). The replacement of egg-yolk phosphatidylcholine to dipalmitoylphosphatidylcholine or distearoylphosphatidylcholine did not affect the sensitivity to the peptide at 25 °C (data not shown), indicating that the fluidity of the matrix lipid did not have appreciable influence on the permeability change of liposomes.

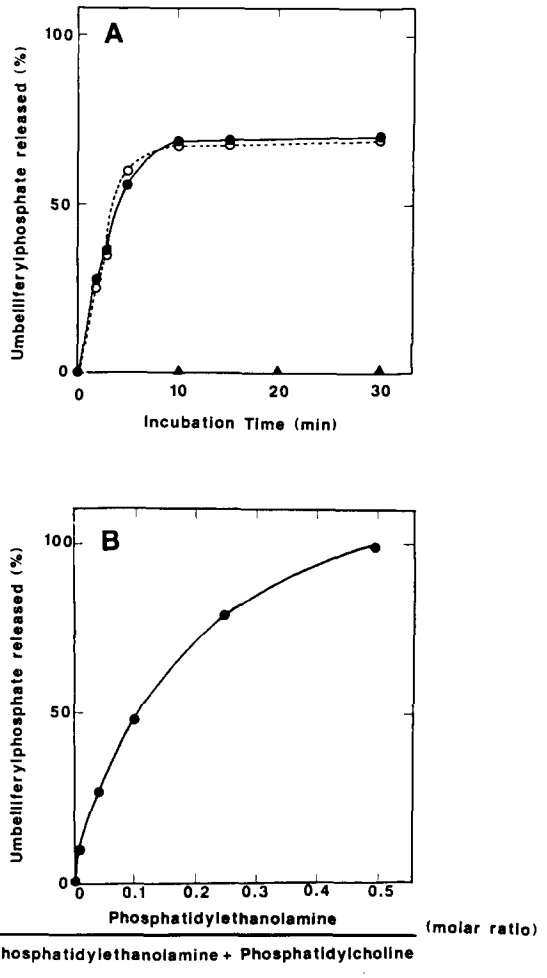


Fig. 2. Effects of temperature and phosphatidylethanolamine content on marker release from liposomes induced by Ro09-0198. Temperature dependence (A). Liposomes were prepared from egg-yolk phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio, 0.75 : 0.1 : 1.0) with (●, ○) or without (▲) egg-yolk phosphatidylethanolamine at molar ratio of 0.25. Liposomes (10 μM as phospholipids) were incubated with 5 μM Ro09-1098 at 0 °C (○) or 37 °C (●, ▲). The release of umbelliferyl phosphate was measured at the designated times. Effect of phosphatidylethanolamine content (B). Liposomes (10 μM as phospholipids) composed of phospholipids (egg-yolk phosphatidylcholine and egg-yolk phosphatidylethanolamine at various ratio), dicetyl phosphate and cholesterol (molar ratio, 1.0 : 0.1 : 1.0) were incubated with 5 μM Ro09-0198 at 37 °C. After 30 min the release of umbelliferyl phosphate was determined.

*Structural requirements on phosphatidylethanolamine for marker release by Ro09-0198*

The various structural analogs of phosphatidylethanolamine were incorporated into liposomes composed of egg-yolk phosphatidylcholine/dicetyl phosphate/cholesterol, and umbelliferyl phosphate release from these liposomes by Ro09-0198

TABLE I

MARKER RELEASE FROM LIPOSOMES CONTAINING STRUCTURAL ANALOGS OF PHOSPHATIDYLETHANOLAMINE INDUCED BY Ro09-0198

Liposomes were prepared from a structural analog of phosphatidylethanolamine, egg-yolk phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio, 0.25 : 0.75 : 0.1 : 1.0). 10 μM of each of these liposomes was incubated with various concentration of Ro09-0198 at 37 °C. After 30 min the amounts of umbelliferyl phosphate released from liposomes were measured. Values are amounts (μM) of Ro09-0198 required for 50% marker release.

| Structural analog | 50% marker release (μM) |
|---|---|
| Diacylphosphatidylethanolamine | 5 |
| Dialkylphosphatidylethanolamine | 5 |
| 1-Acylglycerophosphoethanolamine | 5 |
| Alkylphosphoethanolamine | > 250 |
| Phosphatidyl-*N*-monomethylethanolamine | > 250 |

was measured (Table I). Alike liposomes containing diacylphosphatidylethanolamine, those containing either dialkylphosphatidylethanolamine or 1-acylglycerophosphoethanolamine were reactive with the peptide. Liposomes containing alkylphosphoethanolamine, however, were insensitive to the peptide. These findings indicate that a hydrophobic moiety linked to glycerol in phosphatidylethanolamine is required for sensitivity to the peptide and that either the number or the mode of linkage of hydrophobic moiety to glycerol is not important. Liposomes containing phosphatidyl-*N*-monomethylethanolamine could not be damaged by the peptide, indicating that a primary amino group is precisely recognized to cause permeability increase.

*Selective release of aqueous markers from liposomes by Ro09-0198*

Various markers with different molecular weights were entrapped into liposomes containing phosphatidylethanolamine and their release from liposomes upon incubation of liposomes with Ro09-0198 was measured (Fig. 3). Glucose ($M_r$ 180) and umbelliferylphosphate ($M_r$ 243) were released increasingly with increase in the concentration of the peptide, whereas sucrose ($M_r$ 342) was only slightly released under the same experimental conditions. Inulin ($M_r$ 5000) could not be released appreciably when liposomes were treated with Ro09-0198. It could be concluded that the permeability increase induced by Ro09-0198 is rather specific to molecules smaller than sucrose.

*Effect of phosphatidylethanolamine content in liposomal membrane on inhibition of Ro09-0198-induced hemolysis*

As reported previously, Ro09-0198 induced hemolysis and the preincubation of the peptide with liposomes containing phosphatidylethanolamine inhibited the hemolytic activity [3]. To explore the effect of phosphatidylethanolamine contents in membranes on the interaction of the peptide with phosphatidylethanolamine, inhibition of Ro09-0198-induced hemolysis by liposomes containing various amounts of phosphatidylethanolamine was examined (Fig. 4). The liposomes inhibited the hemolysis when they contained phosphatidyl-

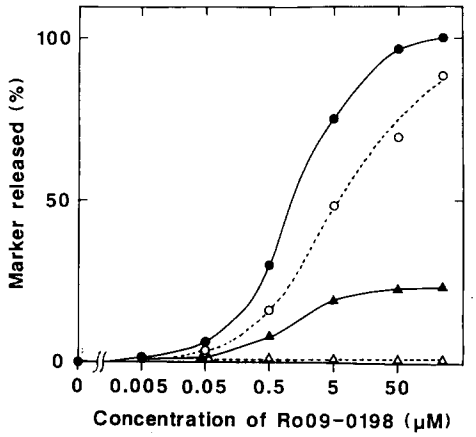


Fig. 3. Release of markers from liposomes induced by Ro09-0198. Liposomes composed of egg-yolk phosphatidylcholine, egg-yolk phosphatidylethanolamine, dicetyl phosphate and cholesterol (molar ratio, 0.75 : 0.25 : 0.1 : 1.0) were swollen in 0.3 M glucose containing [$^3$H]glucose (●), umbelliferyl phosphate (○), [$^{14}$C]sucrose (▲) or [$^3$H]inulin (△), and untrapped markers were removed. The liposomes (10 μM as phospholipids) were incubated with various amounts of Ro09-0198 for 30 min at 37 °C. The releases of entrapped markers from liposomes were determined using an ultrafiltration cell as described in Materials and Methods.

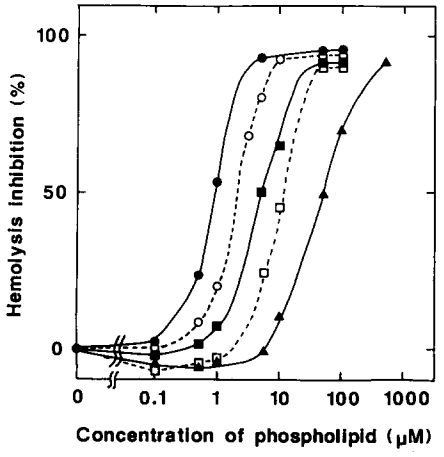


Fig. 4. Effect of phosphatidylethanolamine content on the inhibition of Ro09-0198-induced hemolysis by liposomes. Liposomes were prepared from egg-yolk phosphatidylcholine, egg-yolk phosphatidylethanolamine, dicetyl phosphate and cholesterol (molar ratio, 0.5 : 0.5 : 0.1 : 1.0) (●), (0.75 : 0.25 : 0.1 : 1.0) (○), (0.9 : 0.1 : 0.1 : 1.0) (■), (0.95 : 0.05 : 0.1 : 1.0 )(□) and (0.99 : 0.01 : 0.1 : 1.0) (▲) Ro09-0198 (0.86 nmol) was preincubated with various amounts of these liposomes at 37 °C for 30 min, and the hemolytic activities against human erythrocytes ($1 \cdot 10^7$ cells/ml) by the reaction mixture were measured at 37 °C for 30 min.

ethanolamine, irrespective of the phosphatidylethanolamine content. Although the concentration of phospholipids required for the inhibition was increased with decrease of the phosphatidylethanolamine content in the liposomes, the amount of phosphatidylethanolamine required for the inhibition was about the same.

*Effect of Ro09-0198 on the distribution of phosphatidylethanolamine in liposomal membranes*

A typical ESR spectrum of 12SL-phosphatidylethanolamine in liposomes in the presence of Ro09-0198 is shown in Fig. 5A. An appreciable broadening of the anisotropic resonance lines was observed upon addition of Ro09-0198. On the other hand, the ESR spectrum of 12SL-phosphatidylcholine in the same liposomes was not influenced by addition of Ro09-1098 (Fig. 5B). As a indicator of the broadening, the peak height of the central line ($h/h_0$) was plotted as a function of Ro09-0198 concentration (Fig. 6). The peak height of the central line in ESR spectra of 12SL-phosphatidylethanolamine was decreased gradually with increasing concentration of the peptide. It should be noted that a change in the overall splitting value ($2A_{||}$) was undetectable under the present conditions. Since $2A_{||}$ is known to depend on the mobility of the fatty acyl chain as well as on the spin-spin interaction, the mobility of the acyl chain of phosphatidylethanolamine seemed not to be affected by binding to the peptide. It is also known that the peak height of the central line depends strongly on the interactions between the radicals [23]. The broadening of the spectra by Ro09-0198 suggests that the interaction among radicals is increased by binding of phosphatidylethanolamine to Ro09-0198.

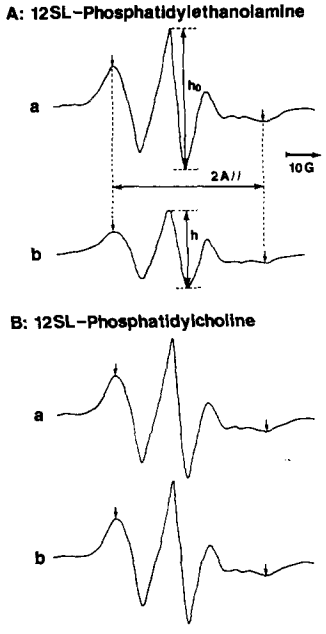


Fig. 5. ESR spectra of liposomes containing 12SL-phosphatidylethanolamine (A) or 12SL-phosphatidylcholine (B) in the presence or absence of Ro09-0198. (A) Liposomes were prepared from egg-yolk phosphatidylcholine, egg-yolk phosphatidylethanolamine, 12SL-phosphatidylethanolamine, dicetyl phosphate and cholesterol (molar ratio, 5:5:0.1::1:10). The liposomes (1 mM as phospholipids) were incubated without Ro09-0198 (a) or with 0.5 mM Ro09-0198 (b) at 37 °C for 30 min. (B) Liposomes were prepared from egg-yolk phosphatidylcholine, egg-yolk phosphatidylethanolamine, 12SL-phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio, 5:5:0.1:1:10). The liposomes (1 mM as phospholipids) were incubated without Ro09-0198 (a) or with 0.5 mM Ro09-0198 (b) at 37 °C for 30 min.

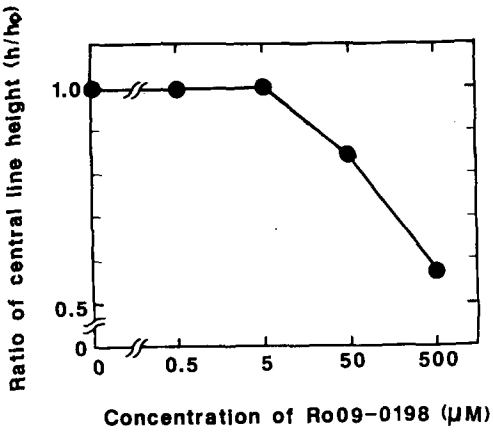


Fig. 6. Dose dependence of Ro09-1098-induced change in central line height of ESR signals. Liposomes prepared as described in Fig. 5A were incubated with various amounts of Ro09-0198 at 37 °C for 30 min. The peak height of the central line in ESR spectrum ($h$) was measured. $h_0$: the peak height of the central line in ESR spectrum of liposomes incubated without Ro09-1098.

## Discussion

In the present work we found that liposomes containing phosphatidylethanolamine were damaged by a cyclic peptide, Ro09-0198, releasing markers trapped in their aqueous compartment.

This peptide also induced an increase in turbidity in suspensions of liposomes containing phosphatidylethanolamine probably due to aggregation of liposomes. Appreciable marker release was induced at lower concentration of Ro09-0198 than that required for change of the turbidity. In our preliminary experiments, similar results were obtained when liposomes containing phosphatidylethanolamine were incubated with duramycin, which is a structural analog of Ro09-0198. Navarro et al. [12] reported that duramycin did not induced leakage of glucose from liposomes with phosphatidylethanolamine at the concentration required for aggregation of liposomes. Although the discrepancy could not be explained at present, the difference in the liposome preparations or in the lot of the antibiotics may be responsible for such difference.

Liposomes containing other phospholipids, such as phosphatidylserine, phosphatidylinositol and cardiolipin, were insensitive to the peptide, indicating that Ro09-0198 can interact specifically with phosphatidylethanolamine, as previously demonstrated by the study on the structural requirements necessary for inhibition of Ro09-0198-induced hemolysis [3]. It was also demonstrated by the hemolysis inhibition study [3] that free amino, phosphate and hydrophobic residues in the molecule are required for binding to the peptide and the distance between an amino and a phosphate group is also important. A glycerol backbone was not necessary for binding, since alkylphosphoethanolamine incorporated into liposomes could bind to the peptide [3]. Liposomes containing alkylphosphoethanolamine was, however, not damaged by the peptide, indicating that the interaction of the peptide with alkylphosphoethanolamine may not be enough for inducing membrane damage. A glycerol backbone of phosphatidylethanolamine seems to be required for causing changes in the physical organization of phosphatidylethanolamine-containing membranes after binding with Ro09-0198.

The permeability increase of liposomes by Ro09-0198 was independent of temperature. The sensitivity of liposomes to the peptide was unaffected by either incorporation of cholesterol or the fatty acid compositions of phospholipids forming the membrane matrix. These findings indicate that the process leading to a permeability increase is not significantly influenced by the fluidity of the matrix membrane.

Ro09-0198 produced a selective permeability change on liposomes containing phosphatidylethanolamine; glucose and umbelliferyl phosphate were effluxed significantly, but sucrose or inulin was not, suggesting that Ro09-1098 may form aqueous 'pores' in the liposomal membrane upon interaction with phosphatidylethanolamine. The size of 'pore' induced by this peptide may have a diameter similar to the hydrodynamic radius of sucrose, a molecule which permeates slightly through liposomes treated with the peptide. The 'pore'-like structure may be formed by 'clustering' of phosphatidylethanolamine molecules on membranes, as observed with interaction of polyene antibiotics with cholesterol in membranes [24]. The Ro09-0198-induced line broadening of the ESR signals of spin-labeled phosphatidylethanolamine is indicative of augmentated interaction between phosphatidylethanolamine molecules by binding with the peptide. A clustering of phospholipids with a spin label at the acyl chain is shown to result in the appearance of a single broad resonance line [25,26]. Such a single broad line could not be observed during the reaction between phosphatidylethanolamine in liposomes and Ro09-0198, indicating that an extensive clustering of phosphatidylethanolamine is not induced by the peptide under the present conditions.

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