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Hemolytic activity of a cyclic peptide Ro09-0198 isolated from *Streptoverticillium*

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Ro09-0198, a cyclic peptide isolated from culture filtrates of *Streptoverticillium griseoverticillatum*, induced lysis of erythrocytes. Preincubation of the peptide with phosphatidylethanolamine reduced the hemolytic activity, whereas other phospholipids present in erythrocytes in nature had no effect. A study of the structural requirements on phosphatidylethanolamine necessary for interaction with the peptide indicates that Ro09-0198 recognizes strictly a particular chemical structure of phosphatidylethanolamine: dialkylphosphoethanolamine as well as 1-acylglycerophosphoethanolamine showed the same inhibitory effect on hemolysis induced by Ro09-0198 as diacylphosphatidylethanolamine, whereas phosphoethanolamine gave no inhibitory effect. Neither phosphatidyl-*N*-monomethylethanolamine nor alkylphosphopropanolamine had an inhibitory effect. Consequently, the hydrophobic chain is necessary for the interaction and the phosphoethanolamine moiety is exactly recognized by the peptide. Ro-09-0198-induced hemolysis was temperature-dependent and the sensitivity of hemolysis differed greatly among animal species.

Introduction

Ro09-0198 has been isolated from culture filtrates of *Streptoverticillium griseoverticillatum* during the screening of immunomodulators from microbial cultures. It is a cyclic peptide and has 15 amino acids (molecular weight, 2041) including unusual amino acids such as lanthionine, β-methyllanthionine, lysinoalanine, β-hydroxyaspartic acid and D-phenylalanine [1]. The structure of this peptide was recently determined as shown in Fig. 1 [2]. It has antitumor and antimicrobial activity in vivo through host-mediated reactions [3].

Several peptide antibiotics containing α,β-unsaturated amino acids or related amino acids such as lanthionine have been isolated [4]. Among them, duramycin, produced by *Streptomyces cinnamoneus* forma *azacoluta* [5], is closely related to Ro09-0198. One difference apparent at the present time is the presence of arginine in Ro09-0198, where there is lysine in duramycin. It was shown recently that duramycin inhibits the chloride transporter [6], the proton pump of clathrin-coated vesicles [7] and the $(Na^+ + K^+)$-ATPase [8]. It also makes cells permeable to small molecules [9], and this antibiotic suggested to be active against biological membranes.

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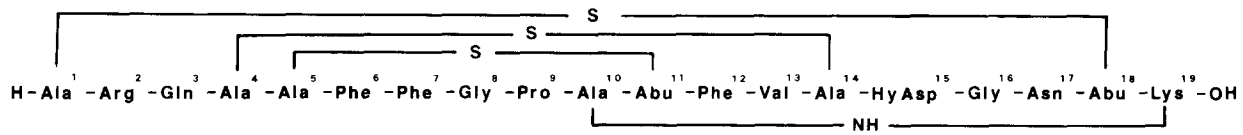

Fig. 1. The structure of Ro09-0198 [2] Abu, aminobutyric acid; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllanthionine; Phe, D-phenylalanine; HyAsp, β-hydroxyaspartic acid.

In the process of studies on biological activities and pharmacokinetics of Ro09-0198, we found that the peptide has an ability to adsorb to phospholipids. In addition, it has been observed that the peptide has hemolytic activity at concentrations higher than that required for showing antitumor activity. In this paper, we describe experiments on the interaction of Ro09-0198 with phospholipids and red blood cells. These studies suggest that the peptide recognizes the particular chemical structure of phosphatidylethanolamine, and can bind to this lipid. The specificity for phosphatidylethanolamine should be valuable for studying the arrangement of this phospholipid in biological membranes. The temperature dependence and difference of the sensitivity of hemolysis among animal species have been also investigated.

Materials and Methods

Materials. Ro09-0198 was isolated from culture broths of *S. griseovertillatum* as described previously [1] and was further purified on a preparative HPLC C-18 column. Phosphatidylethanolamine, phosphatidylcholine and phosphatidic acid from egg yolk were prepared by chromatography on neutral aluminium oxide and silicic acid columns. Phosphatidylethanolamine from *Escherichia coli* was isolated as described previously [10]. [^{14}C]Diacylglycerophosphoethanolamine was prepared from *E. coli* cells cultured in the presence of [1-^{14}C]acetate (Amersham International) as described previously [10]. Cardiolipin from bovine heart was prepared by the method of Faure and Marechal [11]. Bovine brain phosphatidylserine was isolated as described previously [12]. Yeast phosphatidylinositol was isolated by the method of Trevelyan [13]. 1-Acylglycerophosphoethanolamine was obtained from egg-yolk phosphatidylethanolamine by treatment with phospholipase A_2 from snake venom (*Naja naja*). Myristylphosphocholine and dipalmitylphosphatidylcholine were kindly supplied by Drs. Y. Nomura and S. Tsushima (Central Research Division, Takada Chemical Industries, Osaka, Japan). Alkylphosphoethanolamine and alkylphosphopropanolamine were prepared from myristylphosphocholine by means of a transphosphatidylation reaction catalysed by phospholipase D according to the methods of Confurius and Zwaal [14] in the presence of ethanolamine or propanolamine, respectively. Dialkylphosphatidylethanolamine was also prepared from dipalmitylphosphatidylcholine by transphosphatidylation. Sphingomyelin from bovine brain, cholesterol, phosphatidyl-*N*-monomethylethanolamine, dicetylphosphate, stearylamine, phosphoethanolamine and glycerophosphoethanolamine were purchased from Sigma, St. Louis, MO, U.S.A. All lipid preparations showed a single spot on silica-gel thin-layer chromatograms.

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. Blood from rat, rabbit and cow was collected and anticoagulated with heparin. Sheep blood was obtained from Nippon Bio-Supp. Center. Erythrocytes of these animals were isolated in a similar way.

Measurement of hemolysis. Hemolysis was measured using cells labeled with radioactive chromate as described previously [15]. An appropriate amount of Ro09-0198 was dissolved in Tris-buffered saline (600 μl) and placed at the required temperature. The reaction was started by adding 400 μl of ^{51}Cr-labeled erythrocyte suspension ($2.5 \cdot 10^7$ cells/ml). Occasionally, the mixture was

gently shaken during the reaction. After incubation, the reaction mixture was centrifuged at 300 $\times$ g for 5 min, then 400 μl of the supernatant was carefully taken for counting in an auto-well gamma counter (Aloka, JDC 751). The percentage of hemolysis was calculated as follows:

$$\frac{(\text{counts in 400 } \mu\text{l of the supernatant} \times 2.5)}{(\text{counts in } 10^7 \text{ erythrocytes})} \times 100$$

Since it was difficult to measure rapid hemolysis by release of chromate, the time-course of hemolysis from various animal species was assayed by measuring turbidity change. An aliquot (400 μl) of washed erythrocyte suspension ($5 \cdot 10^7$ cells/ml) was transferred to a cuvette containing 1.5 ml of Tris-buffered saline. The cuvette was placed in a double-beam spectrophotometer (Shimazu, UV 140) and equilibrated at the desired temperature. The erythrocyte suspension was agitated continuously with a small magnetic stirrer and the change in turbidity at 675 nm was recorded with time. Then Ro09-0198 dissolved in Tris-buffered saline (100 μl) was added at a final concentration of 5 μM and the mixture was incubated for various periods. The percentage of hemolysis was calculated from the change in turbidity. A control experiment showed that there was no difference between the percentage of hemolysis calculated from the change in turbidity and that calculated using ^{51}Cr-labeled erythrocytes.

Preparation of lipid vesicles. Reverse-phase evaporation vesicles were prepared by the method of Szoka and Papahadjopoulos [16] with a slight modification. These preparations were used as liposomes throughout the present study. The chloroform solution of the lipid mixture (phospholipids 1 μmol, cholesterol 1 μmol and dicetylphosphate 0.1 μmol) was removed under reduced pressure on a rotary evaporator. The lipids were redissolved in 0.6 ml of diethyl ether and 0.1 ml of Tris-buffered saline was added. The resulting two-phase system was sonicated in a bath-type sonicator at 25 °C for about 30 s until the mixture became a homogeneous opalescent dispersion. Diethyl ether was removed under 420 mmHg at 25 °C on a rotary evaporator. After 15 min the material formed a viscous gel and subsequently became an aqueous suspension.

Inhibition of Ro09-0198-induced hemolysis by phospholipids. The phospholipid suspension was prepared by dispersing the dried sample of phospholipids in Tris-buffered saline with a vortex mixer. 0.86 nmol of Ro09-0198 was preincubated with various amounts of either phospholipid suspensions or reverse-phase evaporation vesicles prepared as described above 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 above.

Treatment of erythrocytes with diamide or trypsin. Erythrocytes were treated with diamide according to Haest et al. [17]. Human erythrocytes were washed three times with 154 mM NaCl. 1 vol. of the washed cells was then suspended in 10 vol. of medium A, comprising KCl (90 mM), NaCl (45 mM), Na_2HPO_4/NaH_2PO_4 (10 mM) and sucrose (44 mM). The erythrocytes ($1 \cdot 10^9$ cells/ml) were incubated at 37 °C and pH 8.0 for 30 min under gentle agitation in the presence of 5 mM diamide (Sigma), washed three times and incubated for another 2 h in 10 vol. of medium A without diamide at 37 °C and pH 7.4. After further washing with Tris-buffered saline (pH 7.4), the diamide-treated cells were examined to measure their sensitivity to hemolysis induced by Ro09-0198.

For treatment with trypsin, human erythrocytes were washed three times and suspended in Tris-buffered saline. The washed cells ($1 \cdot 10^9$ cells/ml) were incubated at 37 °C for 30 min under gentle agitation with trypsin (2.5 mg/ml). After washing three times with Tris-buffered saline, cells were subjected to the hemolysis assay induced by Ro09-0198.

Analysis of Ro09-0198–phosphatidylethanolamine interaction by thin-layer chromatography. 25 nmol of [^{14}C]diacylglycerophosphoethanolamine (1.2 nCi/nmol) and 250 nmol of Ro09-0198 were incubated in 0.1 ml of *N*,*N*-dimethylformamide at room temperature for 30 min. The reaction mixture was submitted to thin-layer chromatography (plate: Merck No. 5724) with either *n*-butanol/acetic acid/water (3 : 1 : 1, v/v) or propanol/propionic acid/chloroform/water (3 : 2 : 2 : 1,

v/v) as solvent system. The air-dried plate was exposed to Fuji Ix industrial X-ray films (type 150) for 3 days at −80°C and autoradiography was performed.

Results

Hemolysis of erythrocytes by Ro09-0198

Exposure of washed human erythrocytes to increasing concentrations of Ro09-0198 caused hemolysis progressively at 37°C (Fig. 2A). A Ro09-0198 concentration of 0.8 μM was required for 50% hemolysis, and complete hemolysis was observed at 5 μM. The time-course of hemolysis is also shown in Fig. 2B. The hemolysis occurred rapidly, with short lag time. The time required for 50% hemolysis was 4 min at a peptide concentration of 5 μM.

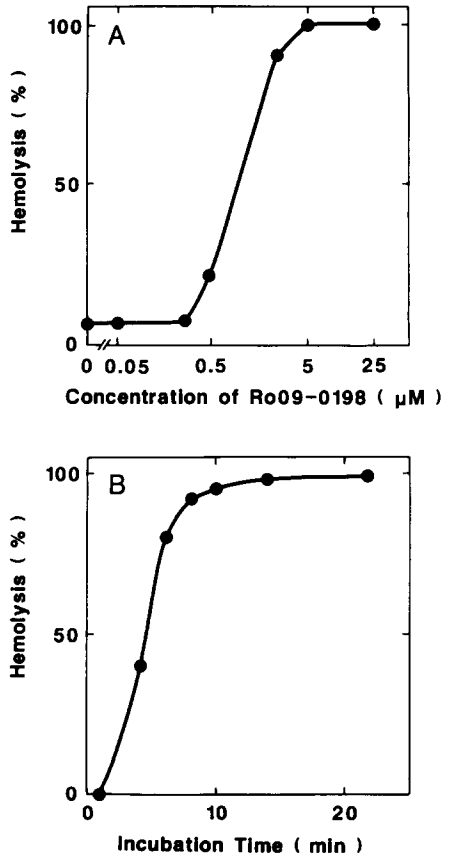

Fig. 2. Hemolysis of human erythrocytes by Ro09-0198. Dose-response (A). Human erythrocytes ($1 \cdot 10^7$ cells/ml) were incubated with various concentrations of Ro09-0198 at 37°C for 30 min. The percentage of hemolysis was determined using ^{51}Cr-labeled erythrocytes as described in Materials and Methods. Time-course (B). Erythrocytes ($1 \cdot 10^7$ cells/ml) were incubated with 5 μM of Ro09-0198 at 37°C and the percentage of hemolysis was measured at various times.

The temperature-dependence of hemolysis was next examined with erythrocytes from various species of animals (Fig. 3). All of the erythrocytes tested were sensitive to the peptide and the rate of lysis of erythrocytes from any animal increased with increasing temperature. The sensitivity of hemolysis, however, differed among animal species, though the contents of phosphatidylethanolamine are similar to each other at about 20–30% of phospholipids in various erythrocytes [18,19]. Rat erythrocytes were most sensitive to Ro09-0198, followed by rabbit, human, cow and sheep erythrocytes. Sheep erythrocytes were the least sensitive to the peptide and the time required for 50% hemolysis was about 2 h even at 37°C. No significant hemolysis occurred at 15%C, even after 5 h.

Inhibition of Ro09-0198-induced hemolysis by phosphatidylethanolamine

To explore the target sites of Ro09-0198 on erythrocytes, inhibitory effects of various kinds of phospholipid on the hemolysis were tested. Ro09-0198 (0.86 nmol) was preincubated with phospholipids, followed by incubation with chromate-labeled human erythrocytes at 37°C for 30 min. As shown in Table I, phosphatidylethanolamine from egg yolk or *E. coli* showed inhibitory effects on hemolysis induced by Ro09-0198, whereas phosphatidylcholine, cardiolipin, phosphatidic acid, phosphatidylinositol, phosphatidylserine or sphingomyelin did not show any inhibition up to 125 nmol. The amount of phosphatidylethanolamine required for 50% inhibition was 0.5 nmol, which corresponds to about a half of the amount of Ro09-0198. Neither phosphoethanolamine nor glycerophosphoethanolamine had effects on the hemolytic activity up to 1 μmol (data not shown).

Reversible binding of Ro09-0198 to phosphatidylethanolamine

When the mixture of Ro09-0198 and radio-labeled phosphatidylethanolamine was developed on thin-layer chromatography with *n*-butanol/

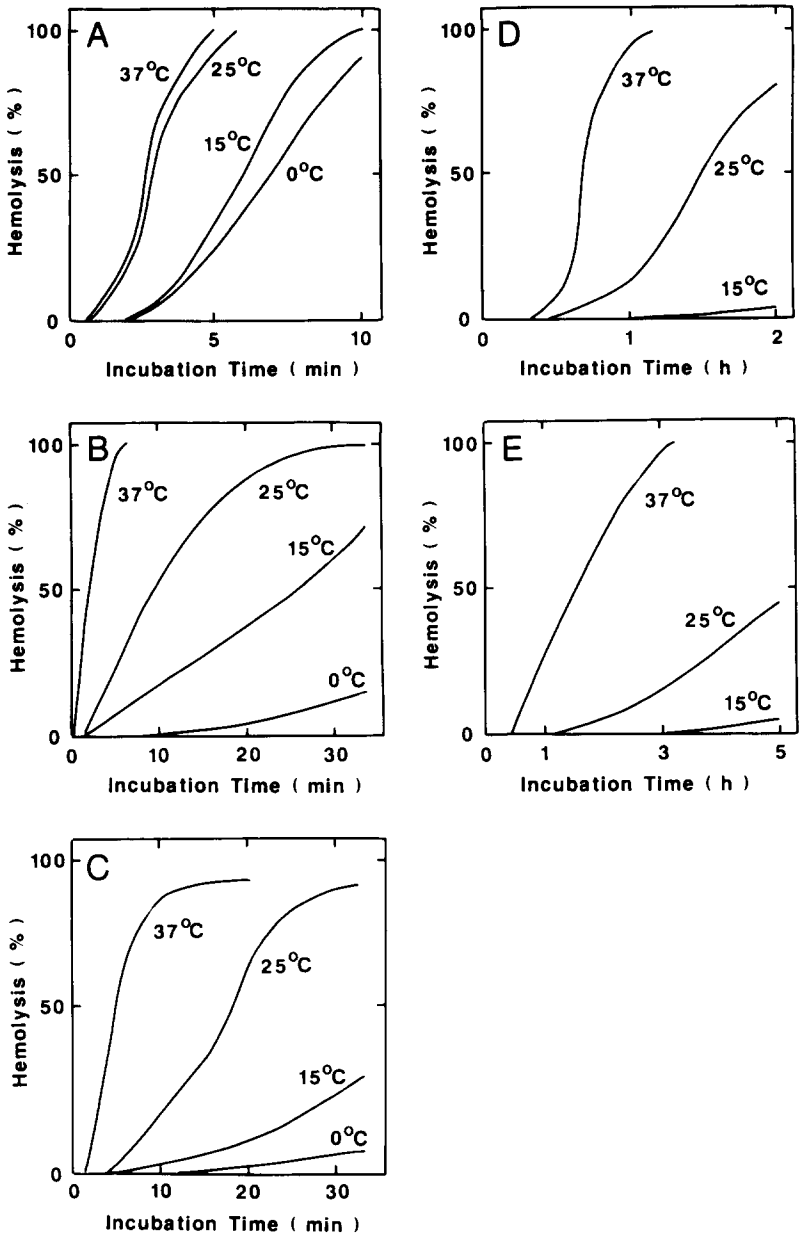

Fig. 3. Temperature dependence of Ro09-0198-induced hemolysis of erythrocytes from various animal species. Erythrocytes ($1 \cdot 10^7$ cells/ml) from rat (A), rabbit (B), man (C), cow (D) or sheep (E) were incubated with 5 μM of Ro09-0198 at 0, 15, 25 or 37° C. The percentage of hemolysis was determined by measuring the turbidity change as described in Materials and Methods.

acetic acid/water (3 ; 1 : 1, v/v) as solvent system, a new radioactive spot (R_F 0.57) was detected besides that of phosphatidylethanolamine (R_F 0.71). Other phospholipids such as phosphatidylcholine, however, did not give a new spot. This spot is assumed to represent the 'Ro09-0198-phosphatidylethanolamine complex'. Ro09-0198 can be bound specifically to phosphatidylethanolamine. A spot corresponding to phosphatidylethanolamine appeared on thin-layer chromatography when 'Ro09-0198-phosphatidylethanolamine complex' was developed in a different solvent system of

TABLE I

INHIBITORY EFFECTS OF VARIOUS PHOSPHOLIPIDS ON HEMOLYSIS INDUCED BY Ro09-0198

Ro09-0198 (0.86 nmol) was preincubated with various amounts of phospholipid suspensions at 37 °C for 30 min. Human erythrocytes ($1 \cdot 10^7$ cells/ml) labeled with ^{51}Cr were added to the reaction mixture and incubated at 37 °C for 30 min. The hemolysis was determined as described in Materials and Methods. The values are amounts (nmol) required for 50% inhibition of hemolysis.

Phospholipids	Origin	50% hemolysis
Phosphatidylcholine	Egg yolk	>125
Phosphatidylethanolamine	Egg yolk	0.5
	E. coli	0.5
Cardiolipin	Bovine heart	>125
Phosphatidic acid	Egg yolk	>125
Phosphatidylinositol	Yeast	>125
Phosphatidylserine	Bovine brain	>125
Sphingomyelin	Bovine brain	>125

propanol/ propionic acid/ chloroform/ water (3 : 2 : 2 : 1, v/v), indicating that 'Ro09-0198-phosphatidylethanolamine complex' can be broken in the presence of chloroform. No covalent bond formation occurs between the peptide and phosphatidylethanolamine.

TABLE II

INHIBITORY EFFECTS OF STRUCTURAL ANALOGS OF PHOSPHATIDYLETHANOLAMINE ON HEMOLYSIS INDUCED BY Ro09-0198

Liposomes were prepared from a structural analog of phosphatidylethanolamine, egg-yolk phosphatidylcholine, dicetylphosphate and cholesterol (molar ratio, 0.25 : 0.75 : 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 mixtures were measured at 37 °C for 30 min. Values are amounts (nmol) required for 50% inhibition of hemolysis.

Structural analog	50% hemolysis
Diacylphosphatidylethanolamine	0.45
Dialkylphosphatidylethanolamine	0.45
1-Acylglycerophosphoethanolamine	0.44
Alkylphosphoethanolamine	0.54
Alkylphosphopropanolamine	> 25
Phosphatidyl-*N*-monomethylethanolamine	>125
Stearylamine	>125

Structural requirements for interaction with Ro09-0198

The various structural analogs of phosphatidylethanolamine were incorporated into liposomes consisting of egg-yolk phosphatidylcholine/ dicetyl phosphate/ cholesterol and subjected to the inhibition assay of hemolysis as described above.

Dialkylphosphatidylethanolamine, 1-acylglycerophosphoethanolamine and alkylphosphoethanolamine showed almost the same inhibitory effect on the hemolysis as diacylphosphatidylethanolamine. Both dipalmitoylphosphatidylethanolamine and dilinoleoylphosphatidylethanolamine showed the same inhibitory effect as phosphatidylethanolamine from egg yolk (data not shown). These findings indicate that a hydrophobic chain is necessary for interaction of the peptide with phosphatidylethanolamine, although an alkyl chain structure is not so strictly recognized. Alkylphosphopropanolamine, phosphatidyl-*N*-monomethylethanolamine and stearylamine were not inhibitory, suggesting that the phosphoethanolamine moiety should be essentially required for the interaction with the peptide. It can be concluded that Ro09-0198 recognizes the particular chemical structure of phosphatidylethanolamine rather than the specific membrane structure formed by assembly of phosphatidylethanolamine such as hexagonal II structure, since 1-acylglycerophosphoethanolamine, which does not have a shape favorable to the formation of hexagonal II structure, was as active as phosphatidylethanolamine.

Preincubation of Ro09-0198 with phospholipid vesicles containing phosphatidylethanolamine at 0 °C showed the same inhibitory effect on hemolysis as observed at 37 °C, indicating that the interaction of the peptide with phosphatidylethanolamine is not affected by temperature.

Effects of pretreatment of erythrocytes with diamide and trypsin on hemolysis

Diamide, an SH-oxidizing agent, is known to oxidize membrane SH groups to disulfide bonds and enhance the transbilayer mobility of phosphatidylethanolamine [17,20,21,22]. In this experiment, diamide or trypsin was used to modify erythrocyte membranes, and the effects of these

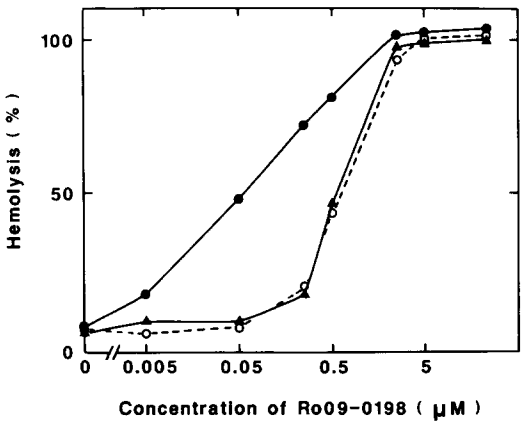

Fig. 4. Effects of pretreatment of human erythrocytes with diamide or trypsin on the hemolysis induced by Ro09-0198. Human erythrocytes ($1 \cdot 10^9$ cells/ml) were preincubated with buffer alone (○), diamide (5 mM) (●) or trypsin (2.5 mg/ml) (▲) at 37 °C for 30 min and washed. The pretreated cells ($1 \cdot 10^7$ cells/ml) were incubated with various concentrations of Ro09-0198 at 37 °C for 30 min. After incubation the percentage of hemolysis was determined as described in Materials and Methods.

membrane modifications on hemolysis induced by Ro09-0198 at 37 °C were examined. Pretreatment with diamide enhanced the sensitivity to hemolysis induced by Ro09-0198 (Fig. 4). The concentration of peptide required for 50% hemolysis was 0.05 μM with diamide-treated cells, and 0.7 μM with non-treated cells respectively. Trypsin treatment, which may destroy (glyco)proteins on the cell surface, showed no effect on the sensitivity of hemolysis to the peptide.

The diamide treatment increased the sensitivity of erythrocytes to Ro09-0198 irrespective of the temperature, since the sensitivity of erythrocytes to the hemolysis at 18 °C was also increased about 10-times by the diamide treatment (data not shown).

Discussion

A cyclic peptide, Ro09-0198, obtained from culture filtrates of *S. griseoverticillatum* has unique characteristics described as follows. (1) The molecule has a high amount of cross-linking, including thioester linkage of lanthionine and β-methyllanthionine as well as an imino bridge of lysinoalanine. This could account for the stability of the peptide. (2) The high content of D-phenylalanine accounts for the hydrophobic property of the peptide. (3) It contains a β-hydroxyaspartic acid, which is contained in vitamin-K-dependent plasma proteins [23,24]. The amino-acid composition of cinnamycin [4], isolated from *Streptomyces cinnamoneus,* is identical to that of Ro09-0198. Although the amino-acid sequence of cinnamycin is still unknown, it is presumed at this time that these two peptides are identical.

We have demonstrated that this rather small peptide recognizes the structure of phosphatidylethanolamine precisely. The peptide can bind to phosphatidylethanolamine incorporated into liposomes as well as phosphatidylethanolamine suspension. Free amino, phosphate and hydrophobic residues in the molecule are required for recognition by the peptide. The distance between an amino group and a phosphate group is also important, since alkylphosphoethanolamine incorporated into liposome could bind to the peptide, whereas alkylphosphopropanolamine could not. A hydrophobic structural component is not strictly required for binding with Ro09-0198, since dialkylphosphatidylethanolamine, 1-acylglycerophosphoethanolamine and even monoalkylphosphoethanolamine showed the same activity as diacylphosphatidylethanolamine.

It was previously proposed that duramycin, a structural analog of Ro09-0198, recognizes a specific membrane conformation formed by phosphatidylethanolamine such as the hexagonal II structure [25]. Ro09-0198 can, however, interact not only with phosphatidylethanolamine but also with 1-acylglycerophosphoethanolamine, which does not adopt a hexagonal II structure, indicating that the present peptide can recognize precisely the chemical structure of the phosphatidylethanolamine molecule.

Ro09-0198 showed hemolytic activity against erythrocytes from various species of animals. The peptide may interact with phosphatidylethanolamine on the membranes, causing hemolysis. The hemolysis by Ro09-0198 was dependent on temperature; a hemolysis rate was increased with increase of the incubation temperature, irrespective of the animal species. The adsorption of Ro09-0198 to liposomes with phosphatidylethanolamine was not affected by the temperature, indicating that

the binding of the peptide with phosphatidylethanolamine is independent of the temperature. The temperature dependence of Ro09-0198-induced hemolysis may be due either to a change in accessibility of membrane phosphatidylethanolamine to Ro09-0198 or to a change in the process leading to hemolysis after binding to phosphatidylethanolamine by temperature. The latter possibility is rather small, since the Ro09-0198-induced liposomal damage was not affected by temperature [26]. The accessibility of phosphatidylethanolamine to Ro09-0198 may reflect the transbilayer distribution of phosphatidylethanolamine or cripticity of phosphatidylethanolamine. Phosphatidylethanolamine may interact with some other components, being hindered from interaction with the peptide, and the interaction between phosphatidylethanolamine and some components may be temperature-dependent. The surface glycoproteins can not be involved in regulating the accessibility of phosphatidylethanolamine, since trypsin treatment of erythrocytes can not affect the sensitivity of erythrocytes to Ro09-0198. Liposomes prepared from the total lipid fraction of erythrocytes could interact with the peptide. These results indicate that accessibility of membrane phosphatidylethanolamine to Ro09-0198 may not be regulated principally by the interaction of phosphatidylethanolamine with some other components.

Phosphatidylethanolamine was supposed to be located mostly in the inner lipid layer of the erythrocyte membranes [27].

It was demonstrated that mild oxidation by diamide of human erythrocytes, which was accompanied by selective cross-linking of the skeletal protein, spectrin, enhanced the susceptibility of phosphatidylethanolamine to exogenous phospholipase A_2 [17,20], probably due to the enhancement of the transbilayer mobility of phosphatidylethanolamine [21,22].

The increase of sensitivity of Ro09-0198-induced hemolysis by diamide treatment may be due to an increase in the amount of phosphatidylethanolamine accessible to Ro 09-0198. The possibility that the increased sensitivity is due to membrane damage induced by diamide itself can not, however, be neglected at present, as pointed out by Frank et al. [28,29].

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