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# Susceptibilities of Phospholipid Membranes Containing Cholesterol or Ergosterol to Gramicidin and its Derivative Incorporated in Lysophospholipid Micelles

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**Complex formation of gramicidin (GA) and desformylgramicidin (des-GA) with sterols was investigated by measuring the intrinsic Trp fluorescence. In organic solvents, the Trp fluorescence of momeric GA was quenched upon binding either cholesterol or ergosterol, but that of monomeric des-GA was not quenched by adding cholesterol. Both dimeric GA and des-GA bound highly to ergosterol, but not to cholesterol, determined by quenching of Trp fluorescence. Furthermore, GA- and des-GA-loaded lysophosphatidylcholine micelles were incubated with phosphatidylcholine vesicles containing cholesterol or ergosterol. The results showed that both monomeric and dimeric peptides hardly bound to cholesterol incorporated into phospholipid vesicles, but markedly bound to ergosterol incorporated into the bilayer membranes. Interestingly, des-GA bound more specifically to the two sterols than GA. In addition, fluorescence resonance energy transfer analysis showed that des-GA bound more specifically to the two sterol than GA.**

**Key words: drug delivery systems, fluorescence, gramicidin, Lysophospholipid, sterol.**

Abbreviations: CMC, critical micelle concentration; des-GA, desformylgramicidin; DMSO, dimethyl sulphoxide; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; eggPC, egg-yolk phosphatidylcholine; FRET, fluorescence resonance energy transfer; GA, gramicidin; LPC, lysophosphatidylcholine; NATA, *N*-acetyltryptophanamide; THF, tetrahydrofuran; TNS, 2-(*p*-toluidino)naphthalene-6-sulphonic acid.

Gramicidin (GA) A is a hydrophobic linear pentadecapeptide produced by *Bacillus brevis* and consists of 15 hydrophobic amino acids: formyl-L-Val[1]-Gly[2]-L-Ala[3]-D-Leu[4]-L-Ala[5]-D-Val[6]-L-Val[7]-D-Val[8]-L-Trp[9]-D-Leu[10]-L-Trp[11]-D-Leu[12]-L-Trp[13]-D-Leu[14]-L-Trp[15]-ethanolamine. Such a unique sequence of alternating L- and D-chirality renders GA sensitive to the environment in which it is placed (*1*). In lipid membranes, GA is capable of forming both single helical dimers (channel forms) with two monomers joined at the N-termini at the bilayer centre and double helical dimers (non-channel forms) in which two monomers are intertwined (*2*). The channel forms are of sufficient length to span the hydrophobic thickness of a lipid bilayer so that they induce selective permeability to monovalent cations in the lipid membranes (*3*, *4*). Conversely, the non-channel forms are usually less than the average thickness of the hydrophobic core of bilayers, so that the conductivity of the non-channel forms is immeasurably low (*4*, *5*). On the other hand, it has been shown that the structure of membrane-associated GA depends on the solvent in which it is dissolved prior to membrane incorporation (*6*, *7*). Therefore, the conformations of GA in membranes are dependent on its 'solvent history'.

GA has broad spectrum activity against gram-positive bacteria, which is mainly related to its ability to selectively facilitate passive diffusion of small cations through cell membranes. Likewise, GA has been found to induce potassium ion leakage from malaria parasite-infected erythrocytes (*8*, *9*). The ability of GA to kill pathogenic microorganisms has recently attracted a great deal of attention because of increased bacterial mutation. The mechanism of action of GA is not entirely understood, but it interacts not with membrane proteins, but with the lipid matrix itself and therefore does not affect mutations of target cells (*10*, *11*). However, there are still many unsolved questions regarding the mechanisms underlying the antimicrobial activity, as well as the mechanisms of resistance evolved by microorganisms against GA. GA is used as an antibiotic, but its toxicity due to hemolytic characteristics limits that application (*12*).

It has been proposed that the toxicity of GA can be reduced considerably by structural modifications (*8*, *13*). Thus, several truncated derivatives of GA such as desformylgramicidin (des-GA) (*14*, *15*) and tryptophan-*N*-formylated GA (*8*) have been designed to gain more insight into the molecular mechanism of GA in membranes. For example, des-GA has little cation conducting channel activity in phospholipid bilayers, while its water conductivity exceeds the permeability of GA by two orders of magnitude (*15*, *16*). On the other hand, it is pharmacologically important to see whether or not GA is selectively more toxic in pathogenic eukaryotic organisms

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than in animal eukaryotes. While cholesterol is the major sterol present in plasma membranes of higher eukaryotes, ergosterol is the major sterol component present in lower eukaryotes such as certain protozoa, yeast and other fungi (*17*). There are several reports about GA-sterol interaction (*18–20*). However, no direct evidence for the complex formation of GA and its derivatives with sterols has been obtained so far.

Micelles are used as membrane mimetics to characterize membrane proteins and peptides. Micelles also offer inherent advantages in fluorescence studies over membranes since they are optically transparent and have well-defined sizes (*21*). Lysophosphatidylcholine (LPC) is not only a single long chain amphiphile, but cone shaped molecule, since the polar head group has a larger cross-sectional area than that of a single acyl chain (*22*). LPC also modifies the function of GA channels in planar bilayers (*22*). The conformation of GA in LPC micelles has been investigated with a variety of techniques (*23*). Incidentally, LPC induces hemolysis at concentrations above the critical micelle concentration (CMC) (*24*). LPC also has several atherogenic actions, including induction of the chemotactic activity of monocytes and macrophage activation (*25*). In addition, the therapeutic efficacy of LPC in experimental sepsis has also been reported (*26*). It has been shown that LPC displays chaperone-like properties (*27*). In addition, recent studies have demonstrated that low-concentration LPC regulates activation of G protein-coupled receptors (*28*). On the other hand, one of the most important properties of drug delivery systems is the release profile, which describes how the drug diffuses out of the carrier after administration. From this viewpoint, LPC has been shown to have excellent penetration enhancing abilities, although it is associated with a high level of local toxicity (*29*, *30*). We have previously demonstrated that LPC micelles adopt a favourable monomeric form of the peptide, since they have a larger hydrodynamic diameter than other surfactant micelles (*31*, *32*). Therefore, we have investigated the difference in the complex formation of peptides with sterols using peptide-loaded LPC micelles.

We first describe the complex formation of GA and des-GA with either cholesterol or ergosterol in connection with the conformation of GA and des-GA by measurement of Trp fluorescence of the peptides in organic solvents. We then discuss the complex formation of GA- and des-GA incorporated in LPC micelles with either cholesterol or ergosterol in phospholipid bilayers by monitoring Trp fluorescence. Finally, we discuss the complex formation of GA and des-GA with either sterol using fluorescence energy transfer analysis.

## MATERIALS AND METHODS

*Materials*—GA A′ (refer to GA) was a natural mixture of 80% GA A, 5% GA B (Trp-11 replaced by phenylalanine residue) and 15% GA C (Trp-11 replaced by tyrosine residue) purchased from Sigma Chemical Co. Cholesterol and ergosterol were from Sigma, and had purities of 99% and ~90%. Ergosterol was recrystallized twice from ethanol. Egg-yolk phosphatidylcholine (egg PC) and *N*-acetyltryptophanamide (NATA) were from Wako Pure Chemical. LPC contained 66% palmitic, 25% stearic, 6% oleic and 1% linolic acid at position 1, with an average molecular weight of 504 (*33*) and a CMC of 7.6 μM (*31*). The eggPC was from Nacalai Tesque, with an average molecular weight of 753 (*33*). The fluorescent probe 2-(*p*-toluidino)naphthalene-6-sulphonic acid (TNS) was purchased from Sigma. Tetrahydrofuran (THF), dimethyl sulphoxide (DMSO) and all other organic solvents were either HPLC or spectroscopic grade. All of the solvents and other reagents were of the highest purity available, and used without further purification.

*Preparation of des-GA*—des-GA was prepared by a modification of the procedure of Killian *et al*. (*14*). GA was modified at the N-terminus by replacing the formyl group with a proton, bearing a net positive charge at neutral pH. To a solution of 500 mg of GA in 40 ml of methanol, 10 ml of 5 M methanolic HCl, freshly prepared by passing hydrogen chloride gas through methanol, was added. The mixture was allowed to stand for 4 h at room temperature. Subsequently, the product was precipitated by addition of large volumes of water, separated by centrifugation at 18,000*g* for 30 min. Then, the peptide was washed with water followed by overnight freeze-drying.

*Tryptophan Fluorescence Spectroscopy*—GA (or des-GA) was dissolved in the appropriate organic solvent (DMSO, methanol or THF). In this experiment, GA was present as a monomer in DMDO or methanol, but as a dimer in THF (*23*). The concentration of GA was calculated from its molar extinction coefficient of 20,700 $M^{-1}$ $cm^{-1}$ at 280 nm (*6*). For des-GA, the same extinction coefficient was used. Fluorescence studies were performed with a JASCO FP-720 spectrofluorometer equipped with a thermostatically controlled cell holder at $30 \pm 0.5$°C. Spectra were obtained at an excitation wavelength of 280 nm and recorded from 290 to 450 nm at 1 nm intervals. Excitation and emission slits with a nominal bandpass of 10 nm were used. Background intensities of samples in which GA was omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ± 1 nm of the ones reported. In addition, NATA was used as a quantum yield standard of Trp, having a value of 0.14 for its quantum yield.

*Fluorescence Quenching Measurements*—For fluorescence quenching experiments with the collisional quencher acrylamide, a peptide concentration of 3.5 μM was used in the appropriate organic solvent. Acrylamide quenching was performed by measuring Trp fluorescence of the peptides after adding aliquots of freshly prepared 5 M acrylamide. Samples were kept in the dark for at least 30 min before measurements. The excitation wavelength used was 280 nm and emission was monitored at 330 nm (in THF) or 344 nm (in DMSO). The acrylamide quenching data were fitted to the following Stern–Volmer equation:

$$\frac{F_o}{F} = 1 + K_{SV}[Q] \quad (1)$$

where $F_o$ and $F$ are the fluorescence intensities at the emission maxima in the absence and presence of

acrylamide, respectively; [*Q*] is the molar acrylamide concentration; and $K_{SV}$ is the Stern–Volmer quenching constant. $K_{SV}$ values were calculated by regression of the initial linear portion of the Stern–Volmer plots.

*Binding of Sterols to GA and des-GA in Organic Solvents by Trp Fluorescence Quenching*—In many cases, the fluorophores can be quenched not only by collision, but also by complex formation with ligands (*34*). The decrease in the Trp fluorescence of GA and des-GA by the addition of either cholesterol or ergosterol was markedly larger than by the addition of acrylamide, as shown in 'RESULTS', implying complex formation between the peptides and sterols. Next, GA (or des-GA) and variable amounts of either sterol were codissolved in the appropriate organic solvent. In this experiment, monomeric GA was prepared by dissolving it in DMSO, where the peptide and sterol were codissolved. The final concentration of GA was kept at 106 μM. The excitation wavelength used was 280 nm and emission was monitored at 334 nm. Fluorescence quenching experiments of Trp residues of GA were carried out in the presence and absence of sterols.

*Preparation of GA- or des-GA-loaded LPC Micelles*—A stock solution in which the peptide was dissolved in the appropriate organic solvent (methanol or THF) at a concentration of 2.7 mM was prepared. LPC was added to the water (1.25 mM). Subsequently, the stock solution of the peptide was added to a LPC dispersion unit and the mixture was sonicated in a bath-type sonifier (Branson Model Sonicator, Yamato) at 50°C for 60 min, so that the final concentrations of the respective peptide and LPC were 49.1 μM and 1.23 mM, corresponding to a GA/LPC molar ratio of 1/25. The concentration of LPC used was about 160-fold that of CMC.

*Preparation of Sterol-containing Phospholipid Vesicles*—EggPC and either cholesterol or ergosterol were codissolved in chloroform, aliquots of the solutions were transferred to sample tubes, and the solvent was completely evaporated at 70°C *in vacuo* for 1 h to give thin, homogeneous films. Subsequently, each sample was hydrated in water and then sonicated at 50°C for 1 h. In this procedure, the dispersions were shown to consist of small unilamellar vesicles, as described in our previous paper (*33*). The final concentration of eggPC in the dispersions was 0.66 mM.

*Complex Formation of GA and des-GA with Sterols in Phospholipid Vesicles by Trp Fluorescence Spectroscopy*—The complex formation of GA and des-GA with either cholesterol or ergosterol was kinetically monitored by the decrease in Trp fluorescence. LPC micelles containing either GA or des-GA and eggPC vesicles containing either cholesterol or ergosterol were separately preincubated at 30°C for 5 min. Then, the eggPC vesicles and LPC micelles, in a 1/4 volume ratio, were mixed and incubated at 30°C for the desired periods. Subsequently, aliquots were taken from the reaction system and used quickly for fluorometry. The final concentrations of GA (or des-GA), sterol, LPC and eggPC in all samples were 39.3, 39.3–118 μM, 0.98 mM and 133 μM, respectively.

*Complex Formation of GA or des-GA with Sterols in Phospholipid Bilayers by Fluorescence Resonance Energy Transfer (FRET)*—Using FRET, the binding of GA and des-GA to sterols in the phospholipid vesicles was judged, according to the procedure described by Onda *et al*. (*33*). In this experiment, FRET was determined by recording fluorescence emission spectra of the Trp residues (a donor) in the presence of TNS (an acceptor). Since the emission spectrum of the Trp residues overlaps the absorption spectrum of TNS, FRET can be used to detect proximity between the peptide and the probe. The efficiency of FRET was determined by the decrease in fluorescence intensities of the Trp residues (at 344 nm) and the increase in fluorescence intensities of TNS (at 437 nm). TNS was codissolved in the sterol-containing organic solvent in the process of vesicle preparation. After the sterol-containing eggPC vesicles and the peptide-loaded LPC micelles were mixed, the mixtures were incubated at 30°C. Then, aliquots were taken from the reaction system and used quickly for measurement of FRET. Under these experimental conditions, all TNS was largely incorporated into the vesicles. Since the absorption of TNS had the minimum value at 288 nm, the Trp residues were excited at 288 nm. The emission was measured from 300 to 570 nm at 30°C. The final concentrations of GA (or des-GA), sterol, TNS, LPC and eggPC in all samples were 39.3, 118, 6 μM, 0.98 mM and 133 μM, respectively.

## RESULTS

*Conformation of GA and des-GA Inorganic Solvents*—In an organic solvent, GA exists in a conformational equilibrium between different monomeric and dimeric (intertwined double-stranded) species (*7*, *18*). In DMSO as well as methanol, GA is present in a monomeric form, which is most likely a left-handed $\beta^{6.3}$-helical conformation (*35*, *36*). In contrast, GA takes a double-helical conformation in a non-polar solvent like THF (*37*, *38*). On the other hand, a detailed conformation of des-GA in an organic solvent is to date unknown. Figure 1 shows

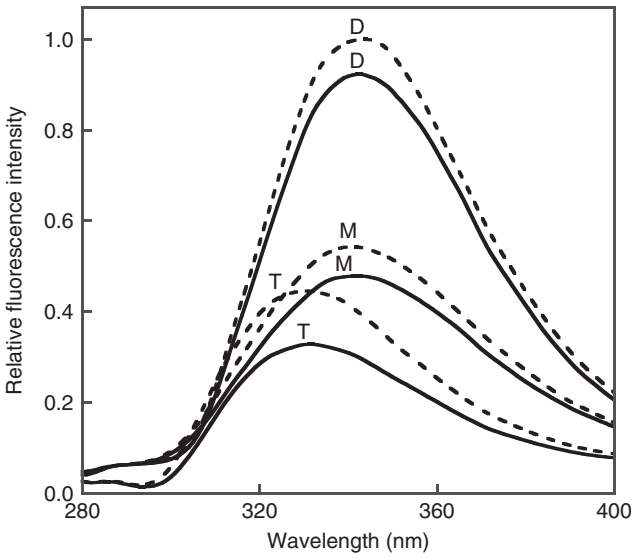


Fig. 1. **The Trp fluorescence spectra of GA (solid curves) and des-GA (broken curves) in DMSO (D), methanol (M) and THF (T).** Trp residues were excited at 280 nm and the emission was recorded at 30°C.

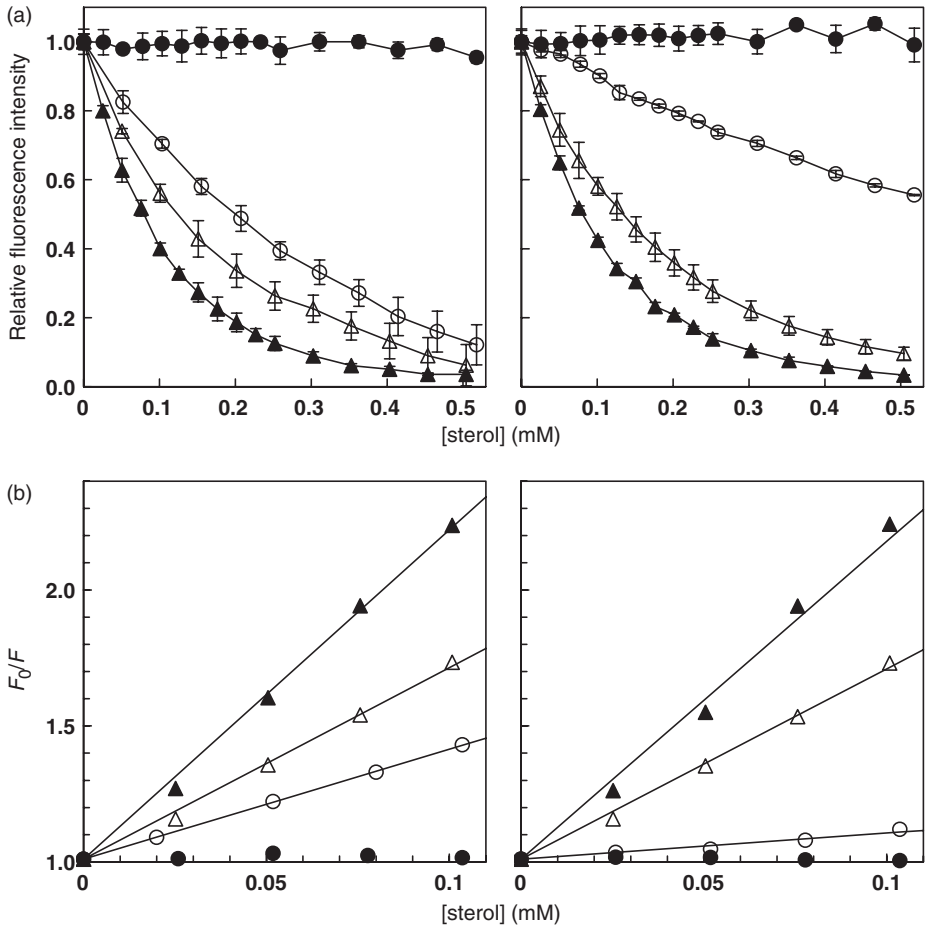


Fig. 2. **Quenching of Trp fluorescence of GA and des-GA by sterols.** (A) Trp fluorescence changes of GA (left) and des-GA (right) in the presence of various concentrations of cholesterol (circles) or ergosterol (triangles) in DMSO (open symbols) or THF (closed symbols). The final concentrations of GA and des-GA were kept at 106 μM. Trp residues were excited at 280 nm and the emission intensity at the peak wavelength was plotted. Standard errors were estimated from multiple measurements and expressed by *error* bars. (B) Stern–Volmer plots of GA (left) and des-GA (right). The same symbols are used as in panel A. $F_o$ and $F$ are the emission maxima in the absence or presence of sterols, respectively. The solid lines represent the best fits of the data to Eq. 1 in the text, and the obtained Stern–Volmer constants are summarized in Table 1.

intrinsic Trp fluorescence spectra of GA and des-GA dissolved in a variety of organic solvents. When excited at 280 nm, NATA in methanol exhibited an emission maximum at 345 nm. The emission maximum of NATA in THF displayed a blue shift at 332 nm with a concomitant increase in fluorescence intensity (data not shown). On the other hand, the respective emission maxima of GA in DMSO, methanol and THF were 344, 343 and 330 nm, respectively, displaying a blue shift in order of solvent polarity. The fluorescent characteristics of des-GA were almost same as that of GA. In contrast to the characteristic of NATA, Trp fluorescence intensities of GA and des-GA decreased significantly upon lowering solvent polarity, consistent with that reported by other researchers (*38*, *39*). Furthermore, the fluorescence intensities of the two peptides were considerably lower than that of NATA, implying that the intra-molecular Trp residues of GA and des-GA interacted with each other.

*Complex Formation of GA and des-GA with Sterols inorganic Solvents*—Melittin, a small hydrophobic linear peptide like GA, produces a quenching of the Trp fluorescence signal by complex formation of a peptide with sterol (*40*, *41*). Recently, similar quenching profiles have been evident for small linear peptides such as pleurocidin (*42*). Unfortunately, no fluorometric evidence for the complex formation of GA and des-GA with a sterol has been obtained. Figure 2A shows plots of Trp fluorescence intensities of GA and des-GA versus the concentrations of sterols in DMSO and THF. Trp fluorescence was markedly quenched after addition of sterol aliquots. Then, the quenching data of the Trp signal were analyzed by a conventional method to distinguish between static and dynamic quenching mechanisms. Dynamic quenching involves a collision between the two molecules with the fluorophore losing energy as kinetic energy, while static quenching involves a more long-lasting formation of a complex between the fluorophore and quencher (*43*). To confirm detailed information for the binding of sterols to either GA or des-GA, the fluorescence quenching constant was estimated by using a Stern–Volmer plot. When dynamic quenching experiments with acrylamide were performed with GA and des-GA, the $K_{SV}$ for

acrylamide was estimated to be $1–3 \times 10^2\,M^{-1}$ by fitting the fluorescence data into the Stern–Volmer relationship. On the other hand, the $K_{SV}$ for cholesterol and ergosterol was estimated to be $7–12 \times 10^3\,M^{-1}$, depending upon the conditions. This value was much greater than that for acrylamide quenching. Additionally, the Stern–Volmer plot for either sterol showed a slightly positive deviation from a straight line. These results suggest that the quenching upon addition of sterol was not dynamic, but static (*i.e.* complex formation) (*44*). Furthermore, the acrylamide quenching experiment was performed in the presence of the peptide and sterol (1:1 molar ratio), resulting in little quenching of the Trp signal, consistent with the results obtained with small peptides such as temporin L (*45*). This result bears out the complex formation of GA and des-GA for sterols.

Figure 2B shows Stern–Volmer plots of the fluorescence intensity as a function of the sterol to peptide molar ratio. For GA and des-GA, the Stern–Volmer plots were linear at sterol concentrations below 0.1 mM, although higher concentrations showed a slightly upward curve. In DMSO, addition of ergosterol resulted in larger quenching of the Trp signal of GA and des-GA than that of cholesterol. Addition of cholesterol caused less quenching of the Trp signal of des-GA than that of GA. On the other hand, the Trp signals of both GA and des-GA in THF were hardly quenched upon addition of cholesterol, while they were highly quenched upon addition of ergosterol. These results show that both monomeric GA and des-GA bound to either cholesterol or ergosterol, although monomeric des-GA only slightly to cholesterol. In addition, both dimeric GA and des-GA bound specifically to the two sterols, *i.e.* two dimeric peptides bound highly to ergosterol, but did not bind to cholesterol. The values of the effective quenching constants, $K_{SV}$, evaluated from the initial slope of the Stern–Volmer plots, are summarized in Table 1.

*Conformation of GA and des-GA in LPC Micelles*—When added from a particular organic solvent to lipid micelles, GA initially becomes associated with the lipid in a conformation that is dependent on the nature of the solvent used as mentioned earlier. Figure 3 shows the fluorescence spectra of GA and des-GA incorporated into LPC micelles with a methanol and a THF solution as starting solvents in the cosolubilization step. Both GA and des-GA adopted a different conformation when incorporated into the LPC micelles from different organic solvents. Thus, the conformations of the peptides in LPC micelles were dependent on the solvent history. It has also been shown that the fluorescence intensity of GA in polar solvents is roughly the same as that of NATA, whereas in micelles or bilayers more than half of the intensity is lost (*46*). This may be attributed to GA being loosely packed in organic solvents, but tightly packed in micelles and bilayers.

Table 1. **Stern—Volmer constants for quenching of Trp fluorescence of GA by sterols in organic solvents.**

| Peptide | Sterol | Organic solvent | $K_{SV} \times 10^3$ ($M^{-1}$) |
|---|---|---|---|
| GA | Cholesterol | DMSO | 4.0 |
| | Ergosterol | DMSO | 7.0 |
| | Colesterol | THF | ND[a] |
| | Ergosterol | THF | 12 |
| des-GA | Cholesterol | DMSO | 0.96 |
| | Ergosterol | DMSO | 7.0 |
| | Cholesterol | THF | ND[a] |
| | Ergosterol | THF | 12 |

$K_{SV}$ values were estimated by fitting the data in Fig. 2 to Eq. 1 in the text.
[a]ND, not determined because of poor slope resolution.

*Complex Formation of GA and des-GA with Sterols in eggPC Vesicles*—A considerable quenching of the Trp signal was observed by complex formation of the peptide with the sterol, as shown in Fig. 2. To monitor the kinetics of the complex formation between the peptides and the sterols, peptide-loaded LPC micelles were mixed with sterol-containing phospholipid vesicles. Figure 4 shows the time-course of the Trp fluorescence intensity when GA- or des-GA-loaded LPC micelles are incubated with either cholesterol- or ergosterol-containing eggPC vesicles, or with sterol-free eggPC vesicles, at 30°C. Trp fluorescence was not quenched upon mixing with sterol-free phospholipid vesicles in a control experiment. When monomeric GA in LPC micelles from methanolic solution was mixed with sterol-containing vesicles, Trp fluorescence was hardly quenched by cholesterol, but was significantly quenched by ergosterol during the first few minutes. The quenching became larger as the concentration of ergosterol was raised. The addition of dimeric GA incorporated into LPC micelles from THF solution caused an effect similar to that of monomeric GA. On the other hand, when des-GA incorporated into LPC micelles from methanolic solution as well as THF solution was mixed with sterol-containing phospholipid vesicles; Trp fluorescence was scarcely quenched with cholesterol, but was markedly quenched for ergosterol. Figure 5 shows plots

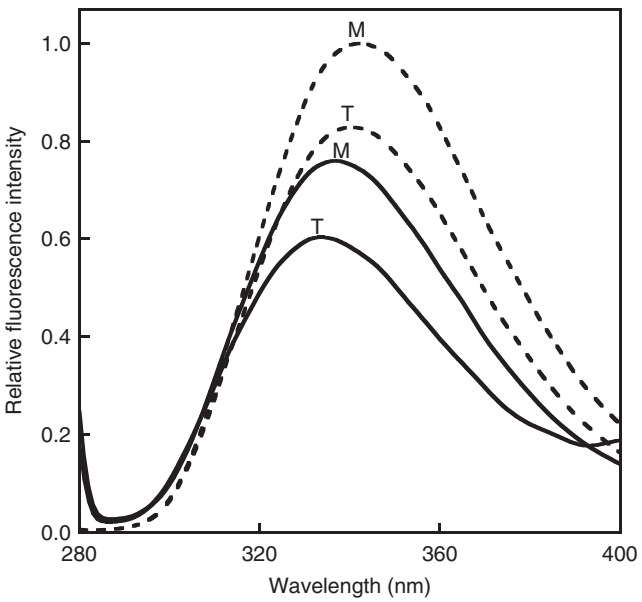


Fig. 3. **Trp fluorescence spectra of GA (solid curves) and des-GA (broken curves) incorporated into lpc micelles.** ga and des-GA were dissolved in methanol (M) or THF (T), and then incorporated into LPC micelles by sonication at 50°C for 30 min. The spectra were recorded at 30°C after the samples were equilibrated at that temperature.

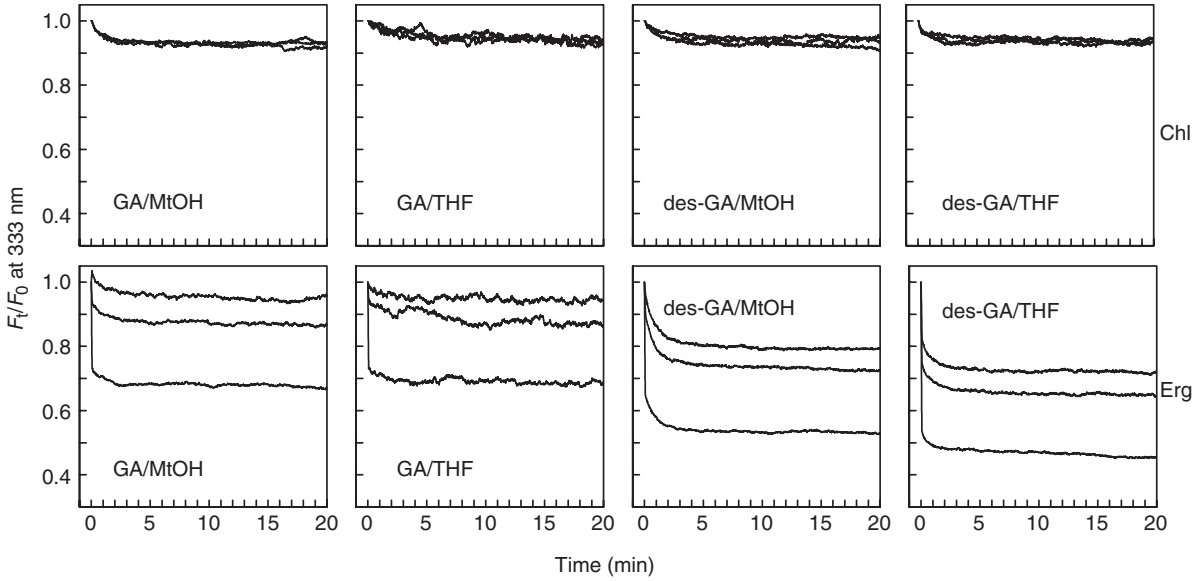


Fig. 4. **Time course of Trp fluorescence change after mixing of GA- or des-GA-loaded LPC micelles with eggPC vesicles containing cholesterol (upper panels) or ergosterol (lower panels).** GA and des-GA were dissolved in methanol (MtOH) or THF (THF), and then the LPC micelles were prepared as described in Fig. 3 and in the text. The curves from the top to bottom in each panel were obtained with molar ratios of sterol to peptide of 0.5, 1, 3, respectively. The final concentrations of GA and des-GA were fixed at 39.3 μM. Trp residues were excited at 280 nm and the emission at 333 nm was monitored at 30°C. $F_t$ is the emission at the incubation time $t$ after the mixing. $F_o$ is the emission when the LPC micelles were mixed with eggPC vesicles that did not contain any sterols.

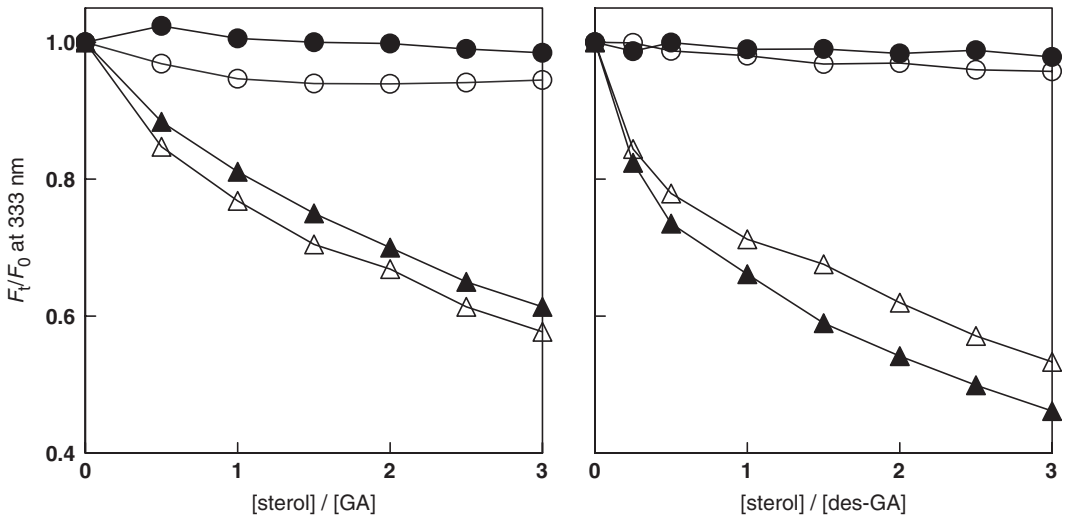


Fig. 5. **Trp fluorescence of the mixture of the LPC micelles and eggPC vesicles in the equilibrium state.** GA (left) and des-GA (right) in methanol (open symbols) or THF (closed symbols) were incorporated into LPC micelles, and then the micelles were mixed with eggPC vesicles containing various concentrations of cholesterol (circles) or ergosterol (triangles) at 30°C. After mixing, Trp fluorescence changes were monitored in the same manner as in Fig. 4, and $F_t/F_o$ values at the incubation time of 15 min, at which the fluorescent decrease is completed, were plotted. The depicted data are the average for multiple measurements.

of the Trp fluorescence intensity in the equilibrium state (at 15 min after the addition of the peptide to the eggPC vesicles) versus the molar ratios of the sterol/peptide derived from Fig. 4. GA and des-GA bound strongly to ergosterol, but not to cholesterol. This result was consistent with that obtained by pleurocidin, a linear peptide like GA (*42*). Interestingly, the binding of des-GA to ergosterol was slightly larger than that of GA, while the binding of des-GA to cholesterol was negligible. Thus, the binding of des-GA was more specific to the two sterols than that of GA. As a whole, characteristics of the binding of GA and des-GA to sterol in the phospholipid membranes were very similar to those in the organic solvents.

*Complex Formation of GA and des-GA with Sterols in Phospholipid Vesicles by FRET*—Figure 6 depicts the time-course of the decrease in Trp fluorescence with concomitant increase in TNS emission due to FRET when

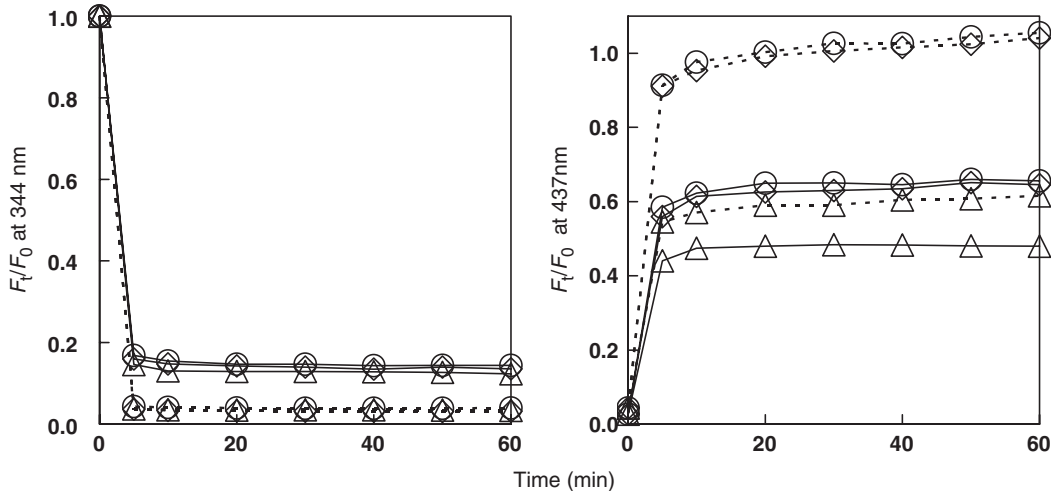


Fig. 6. **Time course of the fluorescence changes of Trp (left) and TNS (right) after mixing of the micelles with the vesicles.** GA (symbols with solid curves) and des-GA (symbols with broken curves) were incorporated into LPC micelles, and then the micelles were mixed with eggPC vesicles containing TNS (diamonds), TNS + cholesterol (circles) or TNS + ergosterol (triangles) at 30°C. The excitation wavelength was 288 nm, and Trp and TNS fluorescence were monitored at 344 and 437 nm, respectively. LPC micelles were prepared from methanolic solution, and essentially the same data were obtained with the micelles prepared from THF.

GA- or des-GA-containing LPC micelles was incubated with sterol-containing eggPC vesicles. GA and des-GA were incorporated into LPC micelles from methanolic solution. After incorporation of GA into the membranes, the Trp signal was quenched by about 20% and the TNS emission was concomitantly increased during the first several minutes. The increase in the TNS emission in the cholesterol-containing vesicles was nearly the same as in the sterol-free vesicles, but much larger than in the ergosterol-containing vesicles. As described in DISCUSSION, TNS fluorescence was decreased by the binding of the peptide to a sterol. Therefore, the results imply that the binding ability of GA to ergosterol is higher than that to cholesterol. Additionally, the intensities of both Trp and TNS reached a steady-state level after the first few minutes. On the other hand, Trp fluorescence almost disappeared after addition of des-GA in LPC micelles. In addition, the TNS signal in the cholesterol-containing vesicles was larger than in the ergosterol-containing vesicles. However, regarding the TNS signal in the cholesterol- and ergosterol-containing vesicles, the des-GA-loaded micelles were obviously larger than the GA-loaded micelles. These results imply that the ability of des-GA to bind sterol is more specific than that of GA, consistent with the results shown in Figs 4 and 5.

## DISCUSSION

When des-GA or GA was transferred from polar to nonpolar solvents, Trp fluorescence was blue-shifted with a concomitant decrease in the quantum yield (Fig. 1). This implies that the conformation of des-GA in organic solvents is likely to be similar to that of GA. It has been proposed that Trp fluorescence is susceptible to self-quenching due to the close proximity between the Trp residues (*47*). If the conformation of des-GA and GA in polar organic solvents is the $\beta^{6.3}$-helical form, Trp-9 and Trp-15 may be close together, leading to quenching of the Trp signal (*39*, *48*). However, such Trp–Trp stacking may become loosely packed in a polar solvent like DMSO without leading quenching of the Trp signal. On the other hand, des-GA and GA in THF preferentially forms double helical conformations with intermolecular Trp–Trp stacking, leading to a pronounced quenching of the Trp signal. Additionally, we expect that in organic solvents the former quenching is less than the latter.

When cholesterol or ergosterol was added to the peptides in organic solvents, Trp fluorescence was markedly quenched. This is the first finding of the complex formation between peptides and the sterols in organic solvents. As seen in Fig. 2B and Table 1, the binding ability of monomeric GA (in DMSO) was greater for ergosterol than for cholesterol. On the other hand, neither the dimeric GA nor des-GA (in THF) exhibited any affinity for cholesterol, while they had much higher affinity for ergosterol. We propose that Trp fluorescence may be quenched by a stacking interaction between the steroid skeleton of sterol and indole rings of the peptide by van der Waals interaction, as described below. The chemical structure of ergosterol differs from that of cholesterol in having a carbon–carbon double bond conjugated system in the second steroid ring at position $C_7$, a trans double bond at position $C_{22}$ and methylated at position $C_{24}$ (*17*, *42*, *49*). The existence of the double bonds makes its structure more rigid than that of cholesterol (*50*). In addition, the structure of ergosterol is only slightly different from that of cholesterol, since α-OH of cholesterol is present in the out-of-plane angle and is missing a hydrogen atom from $C_8$ (*51*). These structural differences may be the reason why the contraction is greater in ergosterol than in cholesterol. The detail of the complex formation of GA and des-GA with the two sterols in membranes is little understood.

The experiments presented in this study demonstrate that upon incorporating GA- or des-GA-loaded LPC micelles into sterol-containing phospholipid vesicles, the peptides bind to sterols (Figs 4 and 5). The affinity was markedly different with species of the sterols and peptides conformers. However, the affinity of GA and des-GA for the two sterols in the lipid vesicles was similar to that in organic solvents. This is probably attributable to solvent history dependence. On the other hand, LPC molecules in micelles are immediately incorporated into phospholipid vesicles (*52*). Therefore the partitioning of the peptides and LPC into the membranes is likely to happen simultaneously. However, Trp fluorescence was not quenched when GA-loaded LPC micelles were mixed with sterol-free phospholipid vesicles, implying that Trp fluorescence was not influenced by the intercalation of LPC into the membranes.

Furthermore, the binding of GA to sterol in phospholipid vesicles was performed by FRET. The location, depth, orientation and distribution of the GA Trp residues are markedly different in the channel and non-channel conformations. The GA Trp residues with the channel conformation are clustered at the membrane-water interface (*39*, *53*), while the GA Trp residues with non-channel conformation are embedded in the deeper regions of the membranes (*48*). Therefore the increased accessibility of Trp residues in the non-channel conformation to sterols may be attributed to the relatively shallow location of Trp-13 and Trp-15 in the membrane, as opposed to the interfacial location of the Trp residues in the channel conformation, leading to the self-quenching of Trp fluorescence (*53*). On the other hand, the membrane probe TNS is located at 1.5–1.8 nm from the bilayer centre, well within the polar head group region (*54*). Thus, TNS molecules are oriented with a charged sulphonic acid group anchored at a shallow location, with the aromatic portion extending toward the hydrocarbon part of the bilayer. Both sterols are deeply located in the bilayer so that the hydroxyl group of sterols is in the immediate vicinity of the phospholipid ester carbonyl, while the hydrophobic parts of sterols are oriented into the bilayer centre (*49*). Thus, it is assumed that the insertion of the peptides into the vesicles first occurs in a spontaneous and very fast process so that Trp fluorescence is immediately quenched by the FRET between Trp as the donor and TNS as the acceptor, as shown in Fig. 6. The peptide-probe complex is then followed by lateral diffusion, leading to a segregation of the probe from the complex in the membranes. Finally, the peptide binds to the sterol in the membranes, resulting in a decrease in TNS fluorescence. In fact, the recovery of Trp fluorescence does not occur because of the quenching by the complex formation between the peptide and the sterol. If the peptide-probe complex does not segregate because of the sterol's weak affinity to the peptide, large TNS fluorescence may be found due to FRET. Therefore, the TNS fluorescence in Fig. 6 suggests that the lower the fluorescence, the stronger the binding affinity of the sterol to the peptide.

In conclusion, the mechanism underlying the complex formation of GA and des-GA with cholesterol and ergosterols was revealed by the peptide uptake from peptide-loaded LPC micelles into sterol-containing phospholipid vesicles. Regarding Trp fluorescence characteristics, monomeric GA and des-GA exhibited higher affinity for ergosterol than for cholesterol, while dimeric GA and des-GA hardly exhibited any affinity for cholesterol, but much higher affinity for ergosterol. Especially, des-GA bound more specifically to the two sterols than GA.

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