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Cinnamycin (Ro 09-0198) Promotes Cell Binding and Toxicity by Inducing Transbilayer Lipid Movement*

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Cinnamycin is a unique toxin in that its receptor, phosphatidylethanolamine (PE), resides in the inner layer of the plasma membrane. Little is known about how the toxin recognizes PE and causes cytotoxicity. We showed that cinnamycin induced transbilayer phospholipid movement in target cells that leads to the exposure of inner leaflet PE to the toxin. Model membrane studies revealed that cinnamycin induced transbilayer lipid movement in a PE concentration-dependent manner. Re-orientation of phospholipids was accompanied by an increase in the incidence of β-sheet structure in cinnamycin. When the surface concentration of PE was high, cinnamycin induced membrane re-organization such as membrane fusion and the alteration of membrane gross morphology. These results suggest that cinnamycin promotes its own binding to the cell and causes toxicity by inducing transbilayer lipid movement.

Lipids are asymmetrically distributed in biological membranes. In the eukaryotic plasma membranes, sphingolipids and phosphatidylcholine are mainly located in the outer leaflet, whereas amino phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) reside in the inner membrane. A number of protein toxins utilize plasma membrane lipids as their receptors; *e.g.* cholera toxin binds ganglioside GM1 (1), shiga toxin recognizes glycolipid Gb3 (2), and lysenin shows high affinity for sphingomyelin (3). These receptor lipids are located in the outer leaflet of the plasma membrane.

Cinnamycin (Ro 09–0198) is a 19-amino acid tetracyclic peptide produced by *Streptomyces* species (4). Cinnamycin is a member of the lantibiotics group of toxins. Lantibiotics are bacteriocins that are characterized by the presence of a high proportion of unusual amino acids (5). Whereas many lantibiotics are directed against other Gram-positive species, the action of cinnamycin is not limited to bacterial cells. Lysis of red blood cells has also been observed upon treatment with this toxin (6). Cinnamycin is unique in that the toxin specifically binds PE that is located in the inner layer of the plasma membrane in mammalian cells (7–9). The mechanism of the recognition of inner leaflet lipid by cinnamycin is not well understood. In the present study, we showed that cinnamycin induced transbilayer lipid movement in target cells so that PE was exposed on the outer leaflet of the plasma membrane. Cinnamycin-induced lipid flip-flop was reconstituted in model membranes. Model membrane study revealed that the presence of PE is required to initiate lipid flip-flop by cinnamycin. Fourier transform infrared-polarized attenuated total reflection (FTIR-PATR) spectroscopy demonstrated that the order parameter of the hydrocarbon chain of PE was significantly reduced in the presence of cinnamycin. FTIR-PATR also showed that the re-orientation of PE was accompanied by the β-sheet formation of cinnamycin. When the surface concentration of PE was high, cinnamycin induced membrane re-organization such as membrane fusion and the alteration of gross morphology of the membrane.

MATERIALS AND METHODS

Cells and Reagents—HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was from Nacalai Tesque Inc., Kyoto, Japan. Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phos-

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[1] The abbreviations used are: PS, phosphatidylserine; NBD, 7-nitrobenz-2-oxa-1,3-diazole-4-yl; C6-NBD-PC, 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine; C6-NBD-PE, 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphoethanolamine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; ELISA, enzyme-linked immunosorbent assay; FTIR-PATR, Fourier transform infrared-polarized attenuated total reflection; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; LDH, lactate dehydrogenase; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; *N*-LRh-PE, *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine; *N*-NBD-PE, *N*-(NBD)-dioleoylphosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPC, palmitoyloleoylphosphatidylcholine; POPE, palmitoyloleoylphosphatidylethanolamine; BSA, bovine serum albumin; TBS, Tris-buffered saline; ATR, attenuated total reflection; PBS, phosphate-buffered saline; GM1, Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4GlcCer; GM3, NeuAcα2,3Galβ1,4GlcCer.

phocholine (C6-NBD-PC), 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphoethanolamine (C6-NBD-PE), *N*-(NBD)-dioleoylphosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (*N*-LRh-PE) were from Avanti Polar Lipids (Alabaster, AL). Alexa Fluor 488-conjugated Annexin V and Alexa Fluor 546-conjugated streptavidin were from Molecular Probes (Eugene, OR). Calcein was from Sigma (St. Louis, MO). Cinnamycin (Ro 09-0198) was kindly provided by H. Ishituka (Nippon Roche Research Center, Japan).

Biotinylation of Cinnamycin—Biotinylated cinnamycin was prepared according to Aoki *et al.* (10) with modification. 0.5 ml of 500 μM cinnamycin in 0.1 M $NaHCO_3$ was mixed with 0.5 ml of EZ-Link™ sulfosuccinimidyl 6-(biotinamido)hezanoate (NHS-LC-biotin, Pierce Biotechnology Inc., Rockford, IL) (8.7 mg/ml sterilized water). After 2-h incubation at room temperature, the reaction was terminated by the addition of 0.1 ml of 1 M lysine solution. Biotinylated cinnamycin was purified by reverse-phase high-performance liquid chromatography on a column of Cosmosil 5C18-AR-300 (Nacalai Tesque). The peptide was eluted with a linear gradient of increasing concentration of acetonitrile from 20 to 60% in water in the presence of trifluoroacetic acid.

ELISA Measurement of Binding of Biotinylated Cinnamycin to Various Lipids—The binding of biotinylated cinnamycin to various lipids was measured by ELISA as described previously (3, 11). In brief, 50 μl of lipid (10 μM) in ethanol was added to a well of microtiter plate (Immulon 1, Dynatech Laboratories, Alexandria, VA). After the solvent was evaporated at room temperature, 200 μl of 30 mg/ml bovine serum albumin (BSA) in Tris-buffered saline (TBS, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl) was added to each well. After washing, the wells were incubated with 50 μl of 100 nM biotinylated cinnamycin in TBS containing 10 mg/ml BSA (1% BSA-TBS) for 2 h at room temperature. The bound cinnamycin was detected by incubating with peroxidase-conjugated streptavidin. The intensity of the color developed with *o*-phenylenediamine as a substrate was measured with an ELISA reader (Bio-Rad, model 550 microplate reader), reading the absorption at 490 nm with reference at 630 nm.

Treatment of HeLa Cells with Cinnamycin—Cells grown on glass coverslips were washed with the incubation buffer (10 mM Hepes-NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). Cells were then incubated with 1 μM cinnamycin or biotinylated cinnamycin in the incubation buffer containing Alexa Fluor 488-conjugated Annexin V. At appropriate intervals, cells were fixed and labeled with Alexa Fluor 546-conjugated streptavidin at room temperature. The stained cells were examined under a Zeiss LSM 510 confocal microscope equipped with a C-Apochromat 63XW Korr (1.2 numerical aperture) objective.

Preparation of Lipid Vesicles—Multilamellar vesicles (MLVs) were prepared by hydrating a lipid film with 20 mM Hepes-NaOH (pH 7.4) and vortex mixing. Liposomes were dispersed by a few seconds of sonication in the bath type sonicator. To prepare large unilamellar vesicles (LUVs), MLVs were subjected to three freeze-thaw cycles followed by extrusion through polycarbonate filters with 0.1-μm pore size (Nuclepore, Maidstone, UK) for 25 times using a two-syringe extruder (12).

Measurement of Transbilayer Lipid Movement in Model Membranes—The transbilayer movement of short chain fluorescent lipids C6-NBD-PE and C6-NBD-PC was measured by mixing labeled LUVs with BSA (13). C6-NBD lipids were incorporated into DOPC or POPC vesicles containing various amount of DOPE or POPE. Vesicles were mixed with 400 μg/ml BSA before addition of 2.5 μM cinnamycin at 30 °C. Selective extraction of the labeled lipid by BSA was detected via the decrease in fluorescence due to quenching by BSA (14). The time course of NBD fluorescence was monitored with a fluorometer (JASCO, FP-6500) (λ_{ex} = 475 nm, λ_{em} = 530 nm).

CD Spectra—CD spectra of 100 μM cinnamycin in 10 mM phosphate buffer (pH 7.4) were measured on a Jasco J-720 apparatus using 0.5-mm path-length quarts cell. Results from five scans were averaged.

Transmission IR—Transmission Fourier transform infrared spectroscopy (FTIR) spectra were measured on a Bio-Rad FTS-3000MX spectrometer, using a liquid nitrogen-cooled mercury cadmium telluride (Hg-Cd-Te) detector and Win-IR software. 1 mM cinnamycin in D_2O containing 40 mM Hepes-NaCl (50 μl) was applied between the two CaF_2 windows. A total of 256 scans was collected. Difference spectra were obtained by digitally subtracting solvent spectra.

Fourier Transform Infrared-polarized Attenuated Total Reflection Spectroscopy—Dry-cast films of POPE/cinnamycin were prepared by uniformly spreading an HFIP solution of POPE (5 μmol)/cinnamycin (0.4 μmol) on the surface of a germanium ATR plate (80 × 10 × 4 mm) followed by the gradual evaporation of the solvent. The last trace of solvent was removed by overnight incubation under vacuum. The film thickness estimated from the applied amount of the lipid was 5–6 μm. The lipid film was hydrated with a piece of D_2O-wet filter paper put over the plate for 3 h. FTIR-PATR measurements were carried out on a Bio-Rad FTS-3000MX spectrometer equipped with a Hg-Cd-Te detector and a PIKE horizontal ATR attachment (PIKE Technologies Inc., Madison, WI) and an AgBr polarizer (15). The total reflection number was 10 on the film side. The spectra were measured at a resolution of 2 cm^{-1} and an angle of incidence of 45° and derived from 256 co-added interferograms with the Happ-Genzel apodization function. The dichroic ratio, R, defined by $\Delta A_{\parallel}/\Delta A_{\perp}$, was calculated from the polarized spectra. The absorbance (ΔA) was obtained either as a peak height of the CH_2 symmetric stretching vibration band or as an area of the amino bands. The subscripts $\parallel$ and $\perp$ refer to polarized light with its electric vector parallel and perpendicular to the plane of incidence, respectively. For ATR correction, refractive indexes of 4.003 and 1.440 were used for germanium and the sample film, respectively.

Measurement of Liposome Fusion—Liposome fusion was monitored by measuring the reduction of resonance energy transfer between NBD- and LRh-labeled lipids in lipid vesicles (16, 17). Donor MLVs (100 μM total phospholipids) containing 1 mol % *N*-NBD-PE and *N*-LRh-PE were mixed with 1 mM acceptor MLVs that do not contain fluorescent lipids. 1 μM cinnamycin was added to the mixture, and the time course of liposome fusion was measured by monitoring the increase of NBD fluorescence at 475 nm at 30 °C.

Electron Microscopy—For negative staining, multilamellar vesicles containing a total of 50 nmol of lipids were incubated with 2 μg of cinnamycin for 30 min at 37 °C. The mixture were fixed with 2.5% glutaraldehyde for 1 h at 37 °C and washed with PBS by centrifugation. The suspension was adsorbed on poly-L-lysine-treated, Formvar/carbon-coated copper grids and negatively stained with 4% aqueous uranyl acetate. The specimens were observed in a transmission electron microscope (Hitachi H-7500, Tokyo, Japan) at an acceleration voltage of 80 kV. For immunogold labeling, 50 nmol of liposomes was incubated with 2 μg of biotinylated cinnamycin. Liposomes were then fixed as above, washed in PBS, and blocked with 2% BSA/PBS for 60 min at 4 °C. The fixed specimens were adsorbed on poly-L-lysine/Formvar/carbon-coated nickel grids. The grids were incubated with 5 nm of gold-conjugated anti-biotin IgG for 60 min at room temperature, washed with distilled water, and fixed with 1% glutaraldehyde for 5 min. The fixed grids were negatively stained with uranyl acetate as above. For freeze-fracture electron microscopy, multilamellar liposomes treated with cinnamycin as described above were frozen in a high-pressure freezer (HPM 010, BAL-TEC Inc., Balzers, Liechtenstein). Immediately after freezing, the frozen samples were placed into liquid nitrogen for storage. The samples were then fractured in a freeze-etching machine (BAF 400T, BAL-TEC Inc., Balzers) at −110 °C, replicated by platinum/carbon, collected on Formvar-coated grids, and examined with an electron microscope (Tecnai 10, Philips, Eindhoven, The Netherlands).

Other Methods—The cytotoxicity of cinnamycin was monitored by the release of lactate dehydrogenase (LDH) as described previously (18). Liposome lysis assay was performed as described previously (3) using calcein as a fluorescent marker.

RESULTS

Cinnamycin Induces Transbilayer Lipid Movement Both in Target Cells and in Model Membranes—Cinnamycin is cytotoxic to both mammalian cells (19) and yeast *Saccharomyces cerevisiae* (20). The fact that cinnamycin-resistant Chinese hamster ovary cells have a defect in PE synthesis (19, 21) suggests that the binding of cinnamycin to PE is essential to induce cell lysis. The specific binding of cinnamycin to PE has been studied relative to the ability of cinnamycin to bind other phospholipids (7, 8) or proteins (22). However, the binding of cinnamycin to other plasma membrane lipid components such as glycolipids has not been examined. Therefore, we first measured the binding of cinnamycin to various phospholipids and glycolipids (Fig. 1). Cinnamycin has high affinity for PE as described previously. Phosphatidylserine (PS) gave a slightly positive signal, whereas other phospholipids and glycolipids tested did not bind cinnamycin. The binding of cinnamycin to PE was not dependent on incubation temperature as described previously (10). Incubations of cinnamycin and PE at 4 °C and 37 °C gave similar degrees of specific binding.

Unlike lipid binding, the cytotoxicity of cinnamycin to HeLa

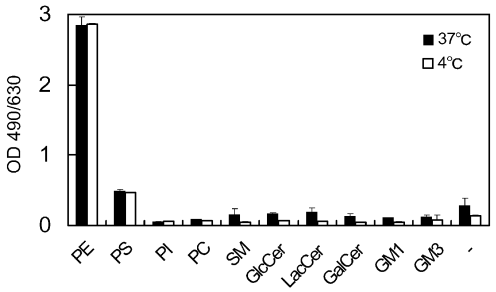

FIG. 1. **Cinnamycin specifically recognizes PE.** Binding of cinnamycin to various lipids was determined by ELISA as described under "Materials and Methods." Data are the mean of duplicate experiments ± difference. When not indicated, difference values are within the *bar*. *PE*, phosphatidylethanolamine; *PS*, phosphatidylserine; *PI*, phosphatidylinositol; *PC*, phosphatidylcholine; *SM*, sphingomyelin; *GlcCer*, glucosylceramide; *LacCer*, lactosylceramide; *GalCer*, galactosylceramide; *GM1*, ganglioside GM1; *GM3*, ganglioside GM3; −, no lipid added.

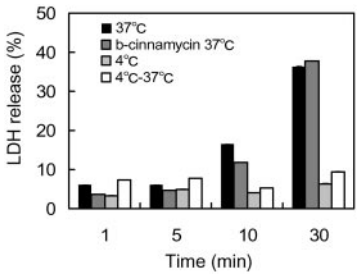

FIG. 2. **High temperature is required for cinnamycin-induced LDH release in HeLa cells.** *37 °C*, HeLa cells were incubated with cinnamycin at 37 °C in the incubation buffer as described under "Materials and Methods." *b-cinnamycin*, cells were incubated with biotinylated cinnamycin for indicated time intervals at 37 °C. *4 °C*, cells were incubated with cinnamycin for indicated time intervals at 4 °C. *4 °C–37 °C*, cells were preincubated with cinnamycin for 30 min at 4 °C. Cells were then washed and incubated for indicated time intervals at 37 °C in the absence of cinnamycin. LDH activity in the medium was measured as described under "Materials and Methods." Data are the mean of duplicate experiments ± difference. When not indicated, difference values are within the *bar*.

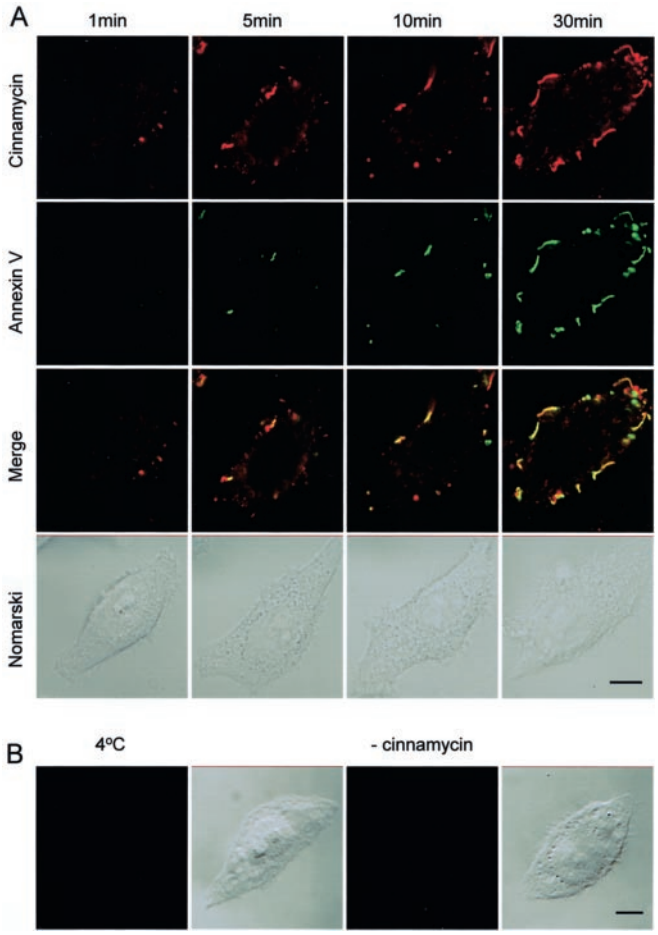

FIG. 3. **Exposure of PE and PS to the cell surface during treatment of HeLa cells with biotinylated cinnamycin.** *A*, cells were treated with 1 μM biotinylated cinnamycin in the presence of Alexa Fluor 488-conjugated Annexin V at 37 °C as described under "Materials and Methods." At appropriate intervals, cells were fixed and labeled with Alexa Fluor 546-conjugated streptavidin. The distribution of Alexa Fluor 546 fluorescence (cinnamycin) and Alexa Fluor 488 fluorescence (Annexin V) were monitored using a Zeiss LSM 510 confocal microscope as described under "Materials and Methods." *B*: *left*, HeLa cells were treated with 1 μM cinnamycin in the presence of Annexin V for 30 min at 4 °C; *right*, cells were incubated in the absence of cinnamycin but in the presence of Annexin V for 30 min at 37 °C. Cells were then fixed and labeled with Alexa Fluor 546-conjugated streptavidin. Merged fluorescence of Alexa 488 and 546 is shown. Very little fluorescence was observed under these conditions. *Bar*: 10 μm.

cells showed a clear temperature dependence (Fig. 2). The toxicity, monitored by the release of LDH activity, increased in a time-dependent manner at 37 °C, whereas at 4 °C LDH was not released. Both naturally occurring and biotinylated cinnamycin showed a similar extent of cytotoxicity as reported earlier (10). Cells treated with cinnamycin at 4 °C, washed and then incubated at 37 °C, did not release LDH. This result indicates that cinnamycin must be present during high temperature incubation. Because cinnamycin binds PE at 4 °C, our result suggests that the amount of PE on the cell surface at steady state was not enough for cinnamycin to induce cytotoxicity. Previously it was shown that the number of cinnamycin bound to the cells increased in a time-dependent manner at 37 °C (10). In Fig. 3, we visualized the binding of biotinylated cinnamycin to HeLa cells at 37 °C. Within 1 min of treatment, cinnamycin gave small positive signal on the cell surface (Fig. 3*A*). The biotin-cinnamycin-positive area increased during incubation. During incubation, Annexin V also became bound to the cell surface. Many of the Annexin V-positive regions were also stained with cinnamycin. Annexin V is known to recognize negatively charged phospholipids such as PS in a calcium-dependent manner (23–26). These results indicate that cinnamycin induces exposure of both PE and PS in HeLa cells at 37 °C. At 4 °C, the binding of cinnamycin to the cells was not detected under fluorescence microscope (Fig. 3*B*), indicating the presence of very few PE molecules on the surface of steady-state cells. Like cinnamycin, Annexin V did not bind cells at 4 °C. In the absence of cinnamycin, Annexin V did not bind cells even at 37 °C (Fig. 3*B*). Although binding of cinnamycin occurs after 1 min of treatment, prolonged incubation was required to induce cell lysis. This result indicates that the transbilayer lipid movement precedes cell lysis.

We then asked whether cinnamycin itself has the ability to induce transbilayer lipid movement in model membranes. In Fig. 4, we measured the transmembrane movement of fluorescent lipids, C6-NBD-PC and C6-NBD-PE, by the method of extraction of short chain lipids by BSA (13, 14). Addition of BSA decreased the fluorescence to 60–70% of initial intensity. This is very likely because of the extraction of the outer leaflet C6-NBD-lipids by BSA (13, 14). The effect of BSA was not changed significantly by the incorporation of POPE into the liposomes. If PE is included in the membrane, the subsequent addition of cinnamycin further decreased the fluorescence. The decrease of fluorescence depends upon the amount of PE in the membrane. Inclusion of as little as 1% PE in the liposomes was effective. Under these conditions liposome lysis monitored by the release of calcein was less than 2%. These results indicate

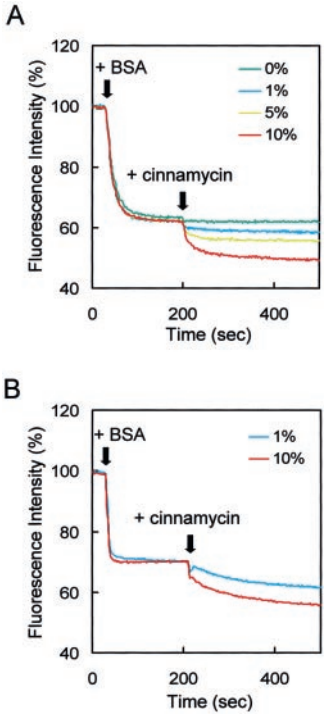

FIG. 4. **Cinnamycin induces transbilayer lipid movement in model membranes.** POPC/POPE LUVs were prepared with the inclusion of 0.25% of short chain fluorescent lipids, C6-NBD-PC (*A*) or C6-NBD-PE (*B*), and the NBD fluorescence was monitored by measuring fluorescence at 535 nm with excitation 475 nm. BSA and cinnamycin were added where indicated. BSA caused a substantial decrease of the fluorescence due to the quenching of extracted lipid. Subsequent addition of cinnamycin induced a further decrease of the fluorescence in PE-dependent manner. Results are representative of three independent experiments.

that cinnamycin promotes the transbilayer movement of the fluorescent phospholipids in model membranes. However, without PE cinnamycin did not induce flip-flop of fluorescent phospholipids. Because cinnamycin induced transbilayer movement of both fluorescent PC and PE, it is suggested that there is no lipid selectivity in the outward translocation of phospholipids.

Re-orientation of Membrane Lipids Was Accompanied by a Structural Change in Cinnamycin—Peptide-induced perturbation of bilayer structures was estimated by examining the CH_2 symmetric stretching band near 2850 cm^{-1} using Fourier transform infrared-polarized attenuated total reflection (FTIR-PATR) (27, 28). The dichroic ratio, R, of an absorption band obtained by PATR spectra is a measure of the orientation of its transition moment or molecular axis. For membranes much thicker (~6 μm) than the penetration depth (under our experimental conditions, 0.2–0.8 μm in the range of 3000–800 cm^{-1}), an R value smaller than 2 indicates that the moment lies essentially parallel to the membrane surface (28–30). From the dichroic ratio R, the frequency and order parameter, S, is calculated by use of Equation 1 (28–30). This parameter is connected to the following,

$$S = -2(R - 2)/(R + 1.45) \quad \text{(Eq. 1)}$$

where the mean orientation angle, α, is between the hydrocarbon chain and the membrane normal through Equation 2, assuming the uniaxial orientation of the chain around the normal,

$$S = (1/2)(3\cos^2\alpha - 1) \quad \text{(Eq. 2)}$$

The results of the calculations are summarized in Table I. The presence of cinnamycin in D_2O-hydrated POPE films significantly reduced the order parameter, indicating that the orientation axis of the lipid was perturbed by the peptide.

We then asked whether the conformation of cinnamycin was altered in the presence of PE. The CD spectrum of cinnamycin gave a minimum at 197 nm, characteristic of a random coil conformation (Fig. 5*A*) (28). The conformation of cinnamycin in solution was also examined using transmission IR (Fig. 5*B*). The major band at 1641 cm^{-1} originated from a random coil structure and was in good agreement with the CD spectrum. Fig. 5*C* shows ATR spectra of D_2O-hydrated DOPE/cinnamycin (12.5/1) films in the region of 1600–1700 cm^{-1}. The band at 1633 cm^{-1} was assigned to β-sheet conformation whereas the band at 1660 cm^{-1} corresponds to the formation of turn structure. The dichroic ratio of the amide I region was close to 2, suggesting a random orientation of the peptide or an orientational angle close to the magic angle (54.7°) with respect to the membrane normal.

Cinnamycin Induces Re-organization of Phosphatidylethanolamine-containing Membranes—When the surface concentration of PE was high, cinnamycin induced dynamic membrane re-organization. In Fig. 6, we measured the activity of cinnamycin to induce liposome fusion. The increase of NBD fluorescence indicates that fusion occurs between donor and acceptor membranes. Cinnamycin-induced membrane fusion in DOPC/DOPE (1:1) and to a lesser degree in POPC/POPE (1:1) liposomes. However PC liposomes did not fuse in the presence of cinnamycin. Fusion requires a high concentration of PE in the membrane, because the addition of 10% POPE to POPC did not cause cinnamycin-induced membrane fusion (data not shown).

The ultrastructure of liposomes in the presence of cinnamycin was examined by negative staining and freeze-fracture electron microscopy (Fig. 7). Cinnamycin did not alter the morphology of DOPC membranes (Fig. 7, *A* and *C*). Addition of 50% DOPE dramatically altered the ultrastructure of the membranes after the treatment with cinnamycin (Fig. 7, *B* and *D*). Characteristic restiform aggregates were accumulated on liposomes. Immunogold labeling showed that cinnamycin was present in these aggregates (Fig. 7*B*). Freeze-fracture images visualized that these structures consisted of moniliform aggregates (Fig. 7*D*).

DISCUSSION

Our results indicate that cinnamycin induces transbilayer lipid movement in target cells. Flip-flop of plasma membrane phospholipids caused the exposure of PE, which is a specific receptor for cinnamycin. The binding of increased number of cinnamycin to the cell surface induced a dramatic membrane reorganization that eventually leads to cell death. We cannot exclude the possibility that the observed binding of cinnamycin was a result of spontaneous flip-flop of cell PE during incubation. However, unlike inward translocation ($t_{1/2}$ = minutes), reported outward translocation of phospholipids is rather slow ($t_{1/2} \cong 1.5$ h) (31–34). In addition, the exposure of PS in treated cells indicates that cinnamycin has the ability to induce transbilayer phospholipid movement. Although cinnamycin weakly bound PS in ELISA, previously it was shown that the binding of cinnamycin to the cells was abolished in the presence of PE-containing liposomes, indicating that cinnamycin specifically recognized PE in the cell membrane (35). Model membrane experiments indicate that PE is necessary for cinnamycin to induce transbilayer lipid movement. Using amino-reactive probe, trinitrobenzene sulfonic acid, it has been shown that in steady-state fibroblasts, 2–2.5% of total PE is exposed to the cell surface (36, 37). We could not detect positive signals with biotinylated cinnamycin at 4 °C incubation under fluorescence microscope. The binding of cinnamycin to PE monitored

TABLE I
Conformations and orientations of lipid hydrocarbon chains

Results are the mean of duplicate experiments ± difference.

Sample[a]	Wavenumber[b]	R^c	S^d	α^e
	cm^{-1}			*degrees*
POPE	2852	1.31 ± 0.01	0.51 ± 0.01	35.1 ± 0.2
POPE/cinnamycin	2853	1.70 ± 0.07	0.22 ± 0.02	46.3 ± 1.0

[a] Samples were hydrated with D_2O.
[b] CH_2 symmetric stretching band.
[c] Dichroic ratio.
[d] Order parameter of the acyl chain.
[e] Mean orientational angle between the hydrocarbon chain and the membrane normal.

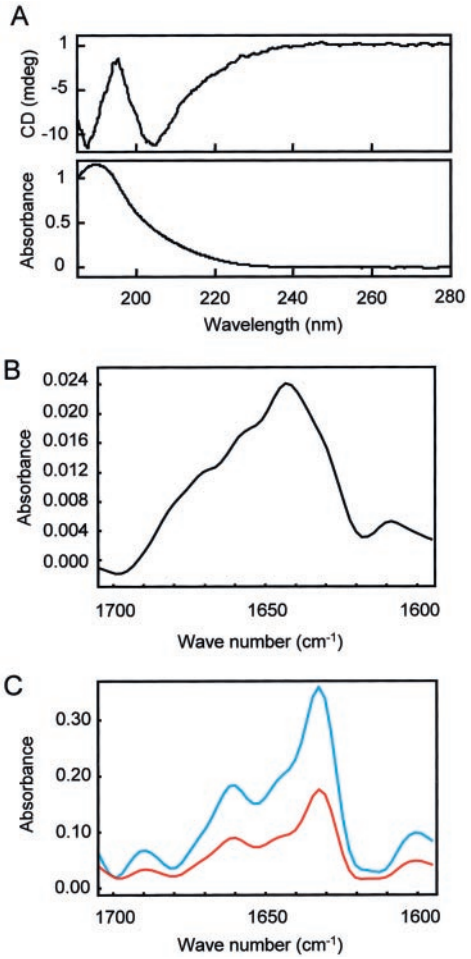

FIG. 5. **The conformation of cinnamycin alters in the presence of PE.** *A*, CD spectrum of cinnamycin in 20 mM Hepes-NaOH (pH 7.4); *B*, FTIR spectrum of cinnamycin in D_2O containing 20 mM Hepes-NaOH (pH 7.4); *C*, FTIR-ATR spectrum of POPE/cinnamycin. The *blue line* denotes $A\|$, whereas the *red line* indicates $A_\perp$ spectrum. Data shown are representative of two independent experiments.

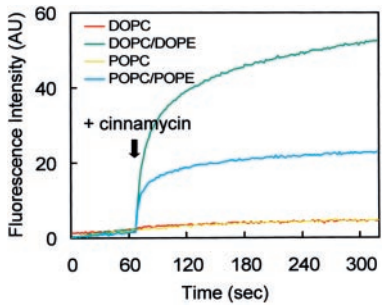

FIG. 6. **Cinnamycin induces fusion of PE-containing membranes.** In donor liposomes, NBD fluorescence was quenched by LRh. Liposome fusion caused the release of resonance energy transfer, which was monitored by the increase of NBD fluorescence. Cinnamycin was added where indicated. Data shown are representative of three independent experiments. Cinnamycin selectively induced fusion of PE-containing membranes.

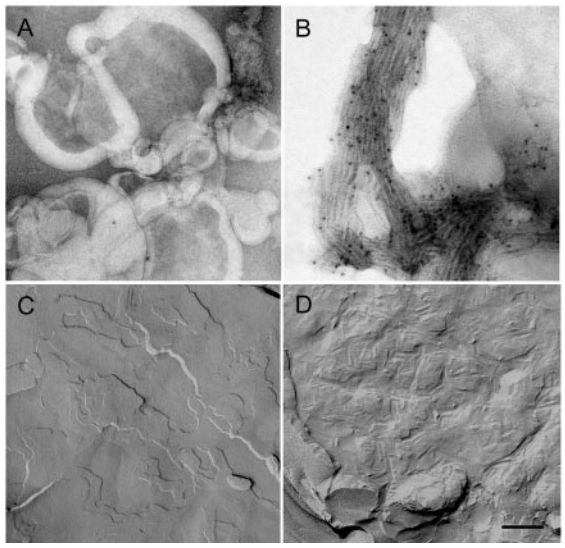

FIG. 7. **Electron micrographs of liposomes treated with cinnamycin.** *A* and *B*, negative staining images; *C* and *D*, frozen replica; *A* and *C*, DOPC in the presence of cinnamycin; *B* and *D*, DOPC/DOPE (1:1) in the presence of cinnamycin. *Images* are representative of two independent experiments with similar results. *Bars*, 100 nm.

by ELISA was not temperature-dependent. These results suggest that the steady-state amount of PE on the cell surface was too low to be detected by biotinylated cinnamycin under fluorescence microscope. However, cinnamycin bound this small amount of PE and did induce transbilayer phospholipid movement. Because preincubation of cells with cinnamycin at 4 °C did not affect the plasma membrane permeability to LDH even after incubation at 37 °C, the binding of cinnamycin to the steady-state amount of cell surface PE was not sufficient to induce membrane damage. Time course experiments indicate that transbilayer phospholipid movement precedes a membrane permeability change. Like cinnamycin, the type I lantibiotic nisin, which is a cationic peptide, induces transbilayer lipid movement without causing membrane leakage (38). Recently Zhang *et al.* (39) showed that natural and synthetic cationic antimicrobial peptides induce lipid flip-flop at peptide concentrations that were 3- to 5-fold lower than those causing leakage of membrane. Induction of transbilayer lipid movement has also been reported with the antimicrobial peptide magainin 2 (40) as well as synthetic peptides (41). Unlike nisin and flip-flop-inducing synthetic peptides, cinnamycin is electrically neutral. Cinnamycin is unique in that flip-flop occurs in PE concentration-dependent manner, and the binding of the toxin to cells is dependent on the transbilayer lipid movement. That is, the toxin self-promotes its own binding to target cells by causing the exposure of the binding PE molecules that

reside in the inner leaflet of the lipid bilayer.

The mechanism of the induction of lipid flip-flop by cinnamycin is not clear. Proton NMR analysis has revealed that one cinnamycin molecule binds one PE (42). Our FTIR study indicates that, in the presence of PE, cinnamycin undergoes conformational change, resulting in the increase of β-sheet structure. Enrichment of β-sheet structure in the presence of phospholipid has also been shown for nisin (43). The observation that the orientation of cinnamycin was random and that the order parameter of acyl chains of PE was significantly reduced suggests that the toxin may locally induce a non-bilayer structure, inducing flip-flop of phospholipids in the cell membrane. This idea is consistent with the fact that, when the membrane concentration of PE is high, cinnamycin could induce dramatic membrane alteration. Recently, using ^{31}P and ^{2}H NMR, Machaidze *et al.* (8) demonstrated a perturbation of the bilayer structure of POPC/POPE (4:1) vesicles in the presence of cinnamycin. Membrane fusion is often accompanied by non-bilayer structure. Our electron microscopy image suggests the formation of non-bilayer structure in cinnamycin-treated PC/PE liposomes. Our results together with the NMR observation by Machaidze *et al.* suggest that the dynamic reorganization of the membrane is associated with cinnamycin-induced cell death.

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