The Membrane-Permeabilizing Effect of Avenacin A-1 Involves the Reorganization of Bilayer Cholesterol

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ABSTRACT Avenacin A-1 is a member of a group of naturally occurring compounds called saponins. It is found in oat plants, where it protects against fungal pathogens. A combined electrical and optical chamber was used to determine the interaction of avenacin A-1 with Montal-Mueller planar lipid bilayers. This system allowed simultaneous measurement of the effect of avenacin A-1 on the fluorescence and lateral diffusion of a fluorescent lipid probe and permeability of the planar lipid bilayer. As expected, cholesterol was required for avenacin A-1-induced bilayer permeabilization. The planar lipid bilayers were also challenged with monodeglucosyl, bis-deglucosyl, and aglycone derivatives of avenacin A-1. The results show that the permeabilizing activity of the native avenacin A-1 was completely abolished after one, two, or all three sugar residues are hydrolyzed (monodeglucosyl, bis-deglucosyl, and aglycone derivatives, respectively). Fluorescence recovery after photobleaching (FRAP) measurements on cholesterol-containing planar lipid bilayers revealed that avenacin A-1 caused a small but significant reduction in the lateral diffusion of the phospholipid probe *N*-(7-nitrobenzoyl-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE). Similarly, with the sterol probe (22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD-Chol), avenacin A-1, but not its derivatives, caused a more pronounced reduction in the lateral diffusion than that observed with the phospholipid probe. The data indicate that an intact sugar moiety of avenacin A-1 is required to reorganize membrane cholesterol into pores.

INTRODUCTION

Resistance to fungal attack in oats was first noted by Goodwin and Pollock (1954), who attributed it to a fluorescent compound referred to as "root tip glycoside." This compound was isolated and shown to comprise four related structures (A-1, A-2, B-1, B-2), collectively known as the avenacins (Maizel et al., 1964; Crombie et al., 1984). They belong to a heterogeneous group of plant triterpene or steroid glycosides called saponins. The structure was elucidated to reveal a trisaccharide-bearing triterpenoid glycoside esterified with *N*-methyl-anthranilic acid (A-1 and B-1), which are fluorescent or benzoic acid (A-2 and B-2). Experiments with other saponins have shown that the fungicidal activity is thought to result from interactions with membrane-bound sterol. Evidence of affinity for membrane sterols comes from electron microscopy studies of saponin-treated membranes, which were found to contain permanent lesions (Seeman, 1974). These lesions are thought to be a micelle-like aggregation of saponins and cholesterol in the plane of the membrane, possibly with the saponin molecules arranged in a ring with their hydrophobic moieties combined with cholesterol around the outer perimeter (Bangham and Horne, 1962; Seeman, 1974). The ability of saponins to cause these lesions makes them hemolytic, and their presence in the diet may increase the permeability of the intestinal mucosa (Johnson et al., 1986).

The carbohydrate residues of avenacin play an important role in neutralizing fungal pathogens (Turner, 1961). These initial findings were confirmed by a number of studies using other saponins and related compounds called glycoalkaloids. Nishikawa et al. (1984) showed that digitonin and its analogs (modified carbohydrate residues) induced hemolysis, activated granulocytes, and caused liposomal membrane damage. The activity was ranked in the following order: digitonin $\geq$ desglucodigitonin $\gg$ glucosyl-galactosyl-digitogenin $>$ galactosyl-digitogenin, digitogenin. Therefore, progressive removal of the sugar residues resulted in a loss of activity. The binding to cholesterol within the liposomal membrane was stoichiometric, and it was suggested that digitonin may form a complex with cholesterol, producing cholesterol-free domains in the membrane. However, it was demonstrated that digitonin could insert into cholesterol-free liposomes without causing disruption. Investigations into glycoalkaloid activity have revealed that similar mechanisms are involved in inducing damage to liposomal membranes. Insertion into the bilayer is followed by sterol-mediated disruption of the membrane. The sugar moiety and the side chain of the sterol at position 24 are also important for membrane-disrupting activity (Keukens et al., 1996).

Although the majority of the studies regarding saponin-induced membrane damage have relied upon the measurement of leakage induced from liposomes, very few have involved measurement of conductance across the membrane. Johnson et al. (1986) showed that the conductivity across the rat gut was increased by saponin treatment, and the effect was dependent on the type of saponin used. Using

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black lipid membranes, Gogelein and Huby (1984) demonstrated that digitonin altered the electrical conductance. This compound induced channel-like fluctuations in both cholesterol-containing and cholesterol-free black lipid membranes by the formation of micellar structures within the lipid lattice. The effects were greater in membranes containing cholesterol. Despite the large number of studies dealing with membrane interactions, only one study to our knowledge has investigated the effects on membrane fluidity by using fluorescence recovery after photobleaching (FRAP). Ishida et al. (1993) demonstrated that treating cultured cardiac cells with digitonin reduced the lateral diffusion of the probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiI) in the plasma membrane. They concluded that large complexes of digitonin and cholesterol are formed in the membrane that obstruct the lateral diffusion of DiI.

To elucidate the effect of avenacin A-1 on the membrane, we have used a technique to simultaneously measure the conductivity and biophysical properties of "solvent-free" Montal-Mueller (Montal and Mueller, 1972) bilayers (PLBs) in the presence and absence of avenacin A-1 (Ladha et al., 1996). The information obtained gives an insight into the mechanisms of saponin-induced membrane reorganization.

MATERIALS AND METHODS

Materials

The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and cholesterol (CHOL) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. The fluorophore 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD-Chol) and *N*-(7-nitrobenzoyl-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE) were purchased from Molecular Probes (Eugene, OR).

Preparation of deglucosylated avenacin A-1 derivatives

Avenacin A-1 was prepared essentially by the method described by Begley et al. (1986), including the two ether precipitation steps. Final purification by thin-layer chromatography (TLC) was carried out using silica G50 plates (Sigma, UK), with chloroform:methanol:water (13:6:1) as the solvent system. Avenacin A-1 was detected by UV visualization. The strongly fluorescent band with the expected Rf value for avenacin A-1 (Crombie et al., 1986) was scraped off the TLC plates, eluted with methanol, and then concentrated by evaporation. A less fluorescent band was also detected with an Rf similar to that previously noted for avenacin B-1 (Crombie et al., 1986). There was no overlap between the two bands.

Mono- and bis-deglucosyl avenacins were prepared from the purified avenacin A-1 by treatment with partially purified avenacinase (Osbourn et al., 1991) (Fig. 1). The reaction products were then separated on TLC as described above. Mono- and bis-deglucosyl avenacins were detected as faster moving fluorescent bands with Rf values identical to those previously described for these compounds (Crombie et al., 1986). The fluorescent bands were then scraped off the TLC plates, eluted with methanol, and then concentrated by evaporation. Enzymatic cleavage could not yield sufficient aglycone, which was subsequently made by treating avenacin A-1 with 0.1 M trifluoroacetic acid for 30 min at room temperature, followed by TLC purification. Estimation of purity for the deglucosylated products was >95%, as determined by visual examination of anisaldehyde/sulfuric acid-treated TLC, fluorimetry, and reverse-phase high-performance liquid chromatography.

Virtually solvent-free planar lipid bilayer formation

The PLBs were prepared as described by Ladha et al. (1996). Briefly, a 25-mm-thick PTFE (polytetrafluoroethylene) septum (Goodfellow, Cambridge, England) with a hole of 200–300 μm diameter in the center was clamped into a specially designed chamber, allowing simultaneous electrical (conductance and capacitance) and FRAP measurements. Before membrane formation, the hole in the septum was coated with 1 μl of 1% (v/v) hexadecane in hexane on each side. The hexane was allowed to evaporate. The PLBs were formed according to the method of Montal and Mueller (1972). To form Montal and Mueller bilayers (PLBs), phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) was added to each side of the chamber, such that the level was above the hole in the septum. Lipid (for FRAP measurements 1 mol% NBD- Chol or NBD-PE was also included) was spread from a hexane solution on the buffer surface in the chambers, and the hexane was allowed to evaporate. The buffer level on one side (*trans* side) was lowered below the hole in the PTFE septum and then raised back to its original level. PLB formation was monitored optically through a microscope and electrically via capacitance measurements.

Conductance measurements

The conductance measurements were carried out as described by Ladha et al. (1996) and Giffard et al. (1996). Membrane potentials were recorded *cis* with respect to *trans*, which was held at ground. Thus a positive potential means that the charge on the *cis* side of the PLB was positive. Membrane current was measured under a voltage clamp of 10 mV with a low-noise operational amplifier connected to a pair of Ag/AgCl electrodes in direct contact with aqueous solutions. Avenacin A-1 and its monodeglucosyl, bis-deglucosyl, and aglycone derivatives were added to the *cis* compartment. The change in the current passing through the PLB was then monitored and logged directly on a computer using SCAN (synaptic current analysis program; John Dempster, University of Strathclyde). To determine the current-voltage (*I-V*) relationship, an applied voltage was ramped over the range $\pm$40 mV at a frequency of 0.01 Hz. All experiments were performed at a controlled room temperature of 23°C.

Lateral diffusion measurements

FRAP was used to measure the lateral diffusion coefficient of the fluorophores, as described by Ladha et al. (1996). After formation of the PLB, a laser beam (λ = 457 nm) was focused on the center of the bilayer. The laser beam was of Gaussian cross-sectional intensity with half-width at $1/e^2$ height of the laser beam at its point of focus equal to 3.3 μm (spot radius). All experiments were performed at a controlled room temperature of 23°C. At least five FRAP measurements were recorded before the addition of avenacin A-1 or its monodeglucosyl, bis-deglucosyl, and aglycone derivatives. After the addition of avenacin A-1 or its derivatives, measurements were taken at regular intervals for the duration of the experiment. Changes in fluorescence induced by avenacin A-1 or its derivatives were monitored using the signal from the photomultiplier before the photobleaching pulse. Ten FRAP curves were collected for every measurement and were averaged before analysis.

Detection of avenacin A-1 in cholesterol-free model membranes

Phospholipid monolayers

To determine whether avenacin A-1 inserts into cholesterol-free phospholipid monolayers, its effects on the monolayer surface tension were deter-

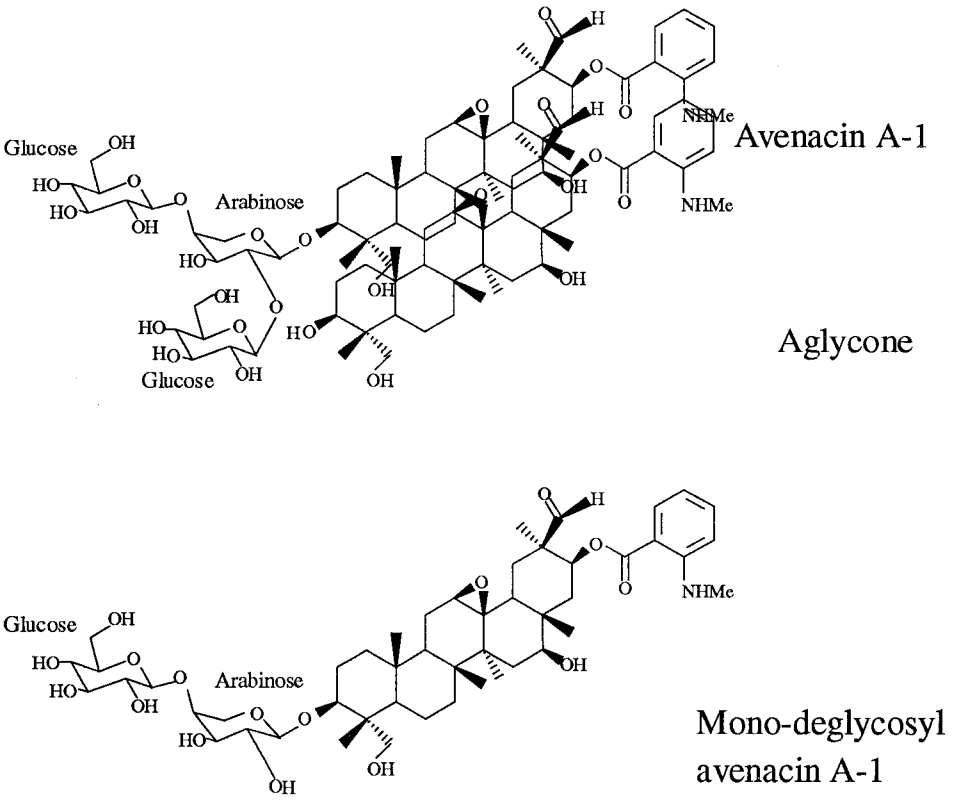

FIGURE 1 Schematic representation of the structure of avenacin A-1 and its deglucosylated derivatives.

mined by the Wilhelmy plate method (Paternotte et al., 1994). Measurements of the behavior of avenacin A-1 at the interface were made with a Langmuir film balance. The balance consisted of a Teflon trough (260 × 115 × 13 mm) with one moving barrier. Surface tension measurements were made using a wetted ground glass Wilhelmy plate. PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) was poured into the trough balance, and 39 nmol of lipid (POPC:DOPE 7:3) was spread on the surface of the PBS buffer in the trough. When the surface tension of the phospholipid monolayer was constant, avenacin A-1 (10 μM final concentration) was added to the subphase. Surface tension measurements were made as a function of time, while the avenacin A-1 adsorbed to the phospholipid interface. All experiments were performed at room temperature (23°C).

Liposomes

To determine whether avenacin A-1 inserts into a cholesterol-free liposomal bilayer, the emission spectrum of 12 mM avenacin A-1 was determined in the absence and presence of liposomes (33 mM phospholipid). Solutions of avenacin A-1 or avenacin A-1 plus liposomes were mixed in a final volume of 3 ml PBS (pH 7.4 filtered through 0.2-μm Millipore membrane). The solutions were all mixed by inversion, and spectra were determined with a spectrofluorimeter (Perkin-Elmer LS-5). The emission spectra were obtained using an excitation wavelength of 357 nm (slit width 2.5 nm) and a measuring emission between 370 and 600 nm (slit width 2.5 nm). The preparation of liposomes was based on the method of Mayer et al. (1986), and only a brief summary will be outlined below. Liposomes consisting of POPC:DOPE (7:3) were prepared. The lipids were placed in a clean round-bottomed flask, dried with argon, and resuspended in 1 ml of PBS (the final concentration of lipid was 13 mM). The suspended lipid was then agitated to produce multilamellar liposomes, gassed with argon, and sealed. The multilamellar mixture was freeze-thawed five times and then pressure-extruded (Lipex Biomembranes) ten times through a Nucleopore drainage disk and two polycarbonate membranes of 100 nm pore diameter. Nitrogen was used to propel the mixture through the extruder with pressures ranging from 200 to 500 psi. Liposomes were gassed with argon, sealed, and stored in the dark at 4°C until required.

RESULTS

Effect of avenacin A-1 on the permeability of the planar lipid bilayer

The increase in PLB conductivity, induced by avenacin A-1, was only observed when cholesterol was present in the membrane. When avenacin A-1 was added to the *cis* compartment of the cell, the conductivity through the lipid PLBs containing 50 mol% of cholesterol increases with time (Fig. 2). The rate at which avenacin A-1 permeabilized the PLB increased with its concentration. At the end of some exper-

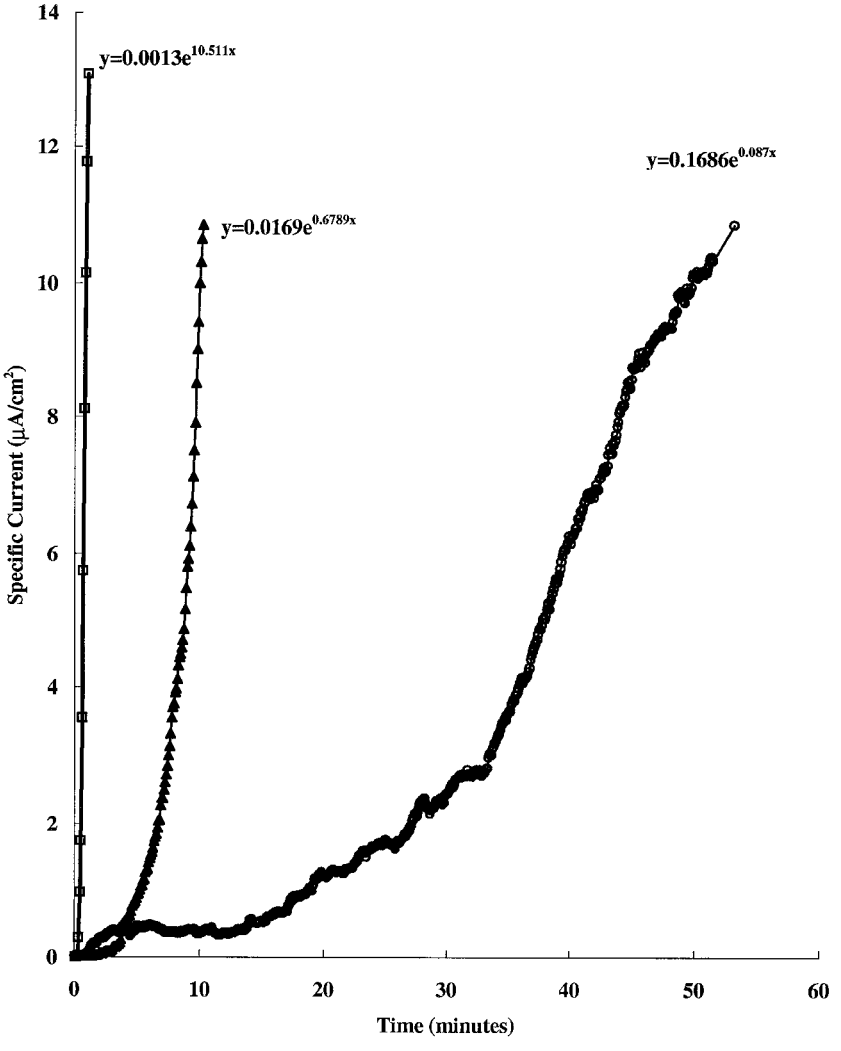

FIGURE 2 The increase in conductivity of POPC:DOPE:CHOL (7:3:10) PLBs after the addition of 0.87 μM (○), 1.23 μM (△), or 2.0 μM (□) avenacin A-1. Avenacin A-1 was added to the *cis* side of the PLB, and a voltage clamp of 10 mV was applied across the bilayer. The equation given for each set of data is a single exponential function ($y = ce^{kx}$, where k is the rate constant) that best represents the data.

iments the PLB was still intact. The current-voltage relationship was determined on these bilayers. As shown in Fig. 3, the current-voltage relationship was linear over the applied voltage range at 0.5 μM (Fig. 3 *A*) and 1.0 μM (Fig. 3 *B*) avenacin A-1. The linear or ohmic current-voltage relationship indicated that there was no voltage dependence (i.e., no exponential increase in current above a threshold voltage, and therefore the pores formed by avenacin A-1 are voltage independent). From these data the number of avenacin A-1 monomers involved per conducting pore was estimated using the theory developed by Vodyanoy et al. (1983) and recently explored by Cosette et al. (1997). The theory states that for pore formers that show voltage-independent behavior (ohmic current-voltage relationship), the number of monomers per conducting pore (N) can be derived as follows:

$$N = \ln(G_2/G_1)/\ln(C_2/C_1)$$

where G_i is the bilayer conductance at a given voltage at avenacin A-1 concentration C_i. Using this relationship, the number of avenacin A-1 monomers per conducting pore was estimated to be 8.3 $\pm$ 1.0.

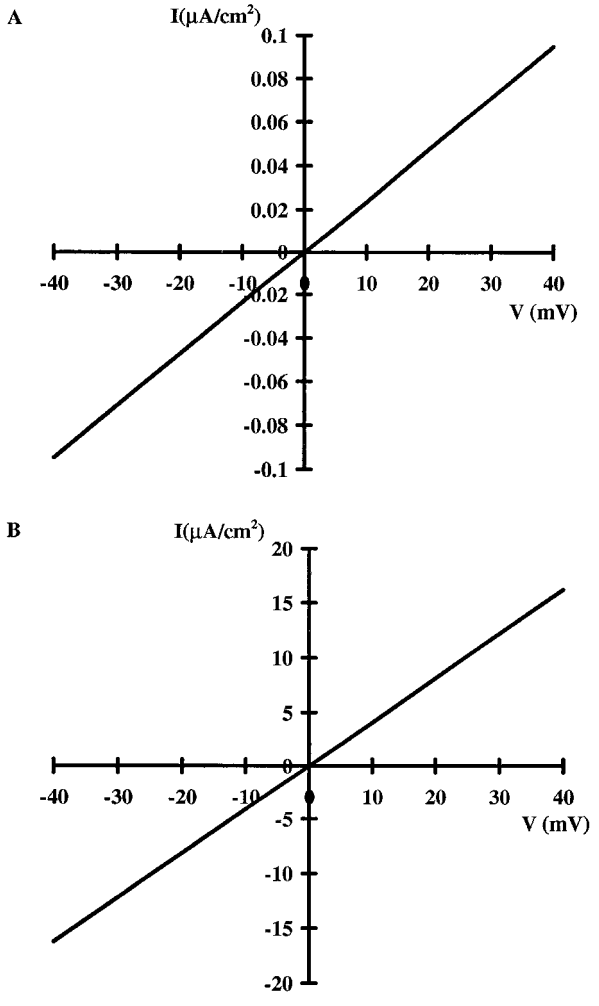

FIGURE 3 Macroscopic current-voltage curve for POPC:DOPE:CHOL (7:3:10) PLBs after equilibration with 0.5 μM (*A*) or 1.0 μM (*B*) avenacin A-1 on the *cis* side.

Effect of removing the sugar moiety on the permeability of the planar lipid bilayer

The planar lipid bilayers were also challenged with monodeglucosyl, bis-deglucosyl, and aglycone derivatives of avenacin A-1. The results reveal that the permeabilizing activity of the native avenacin A-1 was completely abolished (Fig. 4) after one, two, or all three sugar residues were hydrolyzed (monodeglucosyl, bis-deglucosyl, and aglycone derivatives, respectively). Note that in Fig. 4 the *y* scale is 1000 times more sensitive than that of Fig. 2, which makes the data appear noisy. The more sensitive scale was used to amplify the potential differences.

Effect of avenacin A-1 on lateral lipid diffusion within the membrane

As expected (Rubenstein et al., 1979), lateral diffusion values of both fluorescent lipid probes decreased with increasing cholesterol content of the planar lipid bilayer.

On the addition of avenacin A-1, lateral diffusion of NBD-Chol decreased significantly within cholesterol-containing PLBs. This coincided with a decrease in fluorescence and an increase in PLB permeability (Fig. 5). Furthermore, increasing the cholesterol content of the PLB enhanced the avenacin-induced reduction in lateral diffusion (Table 1).

Similarly, with the phospholipid probe NBD-PE, the reduction in lateral diffusion induced by avenacin A-1 was also significant (Table 2), but the magnitude of the decrease was not as large as for NBD-Chol. Although this coincided with an increase in the permeability of cholesterol-containing PLBs, no decrease in fluorescence was observed (data not shown). No changes in lateral diffusion or fluorescence were observed on the addition of avenacin A-1 to planar lipid bilayers containing no cholesterol. This was not due to its the lack of insertion into a cholesterol-free bilayer environment, inasmuch as avenacin A-1 is capable of reducing the surface tension of phospholipid monolayers (Fig. 6). This indicated that avenacin A-1 was inserting into cholesterol-free monolayers. If this is the case, then the fluorescence of avenacin A-1 should increase when it is incorporated into the hydrophobic environment of the cholesterol-free bilayer. This was what was observed from the emission spectra (Fig. 7), which show that there is an enhancement in the fluorescence of avenacin A-1 in the presence of liposomes when compared to the spectra in the absence of liposomes.

The largest reduction in lateral diffusion for both fluorophores was observed when the PLB contained 35 mol% cholesterol. No changes in lateral diffusion or fluorescence of NDB-Chol and NBD-PE (data not shown) were observed on the addition of the monodeglucosyl, bis-deglucosyl, and

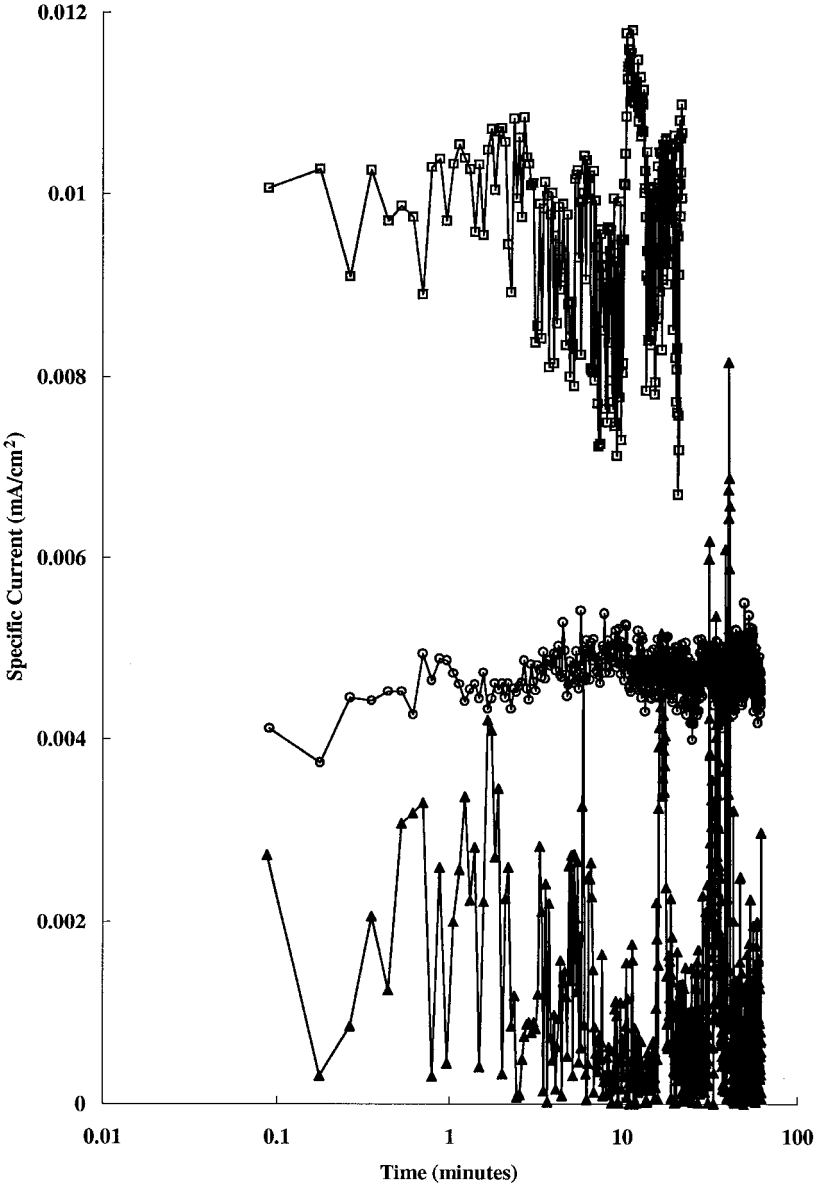

FIGURE 4 The effect on the conductivity of POPC:DOPE:CHOL (7:3:10) PLBs after the addition of monodeglucosyl (○), bis-deglucosyl (▲), and aglycone (□). The derivatives were added to the *cis* side of the PLB (final concentration 2.0 μM), and a voltage clamp of 10 mV was applied across the bilayer.

aglycone derivatives to the PLBs. Under all experimental conditions no immobile domains were detected on the FRAP time scale.

DISCUSSION

In the present study, an attempt has been made to characterize the pore-forming ability of avenacin A-1 and its effects on the lipids within the membrane. The results lead to a model (Fig. 8) for the formation of pores by avenacin A-1. Similar schemes have been put forward by Nishikawa et al. (1984) and Keukens et al. (1996) to represent the membrane disruption caused by saponins and glycoalkaloids.

Step one (Fig. 8 *A*) shows the cholesterol-independent insertion of the aglycone portion of avenacin A-1 into the *cis* leaflet of the membrane. This is supported by the surface tension measurements (Fig. 6) and the fluorescence emission spectra (Fig. 7). However, although avenacin A-1 can insert into cholesterol-free membranes, this does not result in a change in the lateral diffusion coefficient of phospholipids. This suggests that avenacin A-1 can insert into cholesterol-free membranes but does not have the ability to alter the lateral movement of the bilayer phospholipids.

Step two involves the binding of cholesterol to the avenacin A-1 in the membrane (Fig. 8 *B*). Support for this step is demonstrated by the result that there is an absolute requirement for sterol to be present in the PLBs for avenacin A-1 to form pores or induce disruption. This has been shown for a number of glycoalkaloids (Roddick and Drysdale, 1984; Steel and Drysdale, 1988; Keukens et al., 1992) and the saponin digitonin (Nishikawa et al., 1984), by using the liposomal membrane. These results contrast with those of the only other study carried out using PLBs that showed

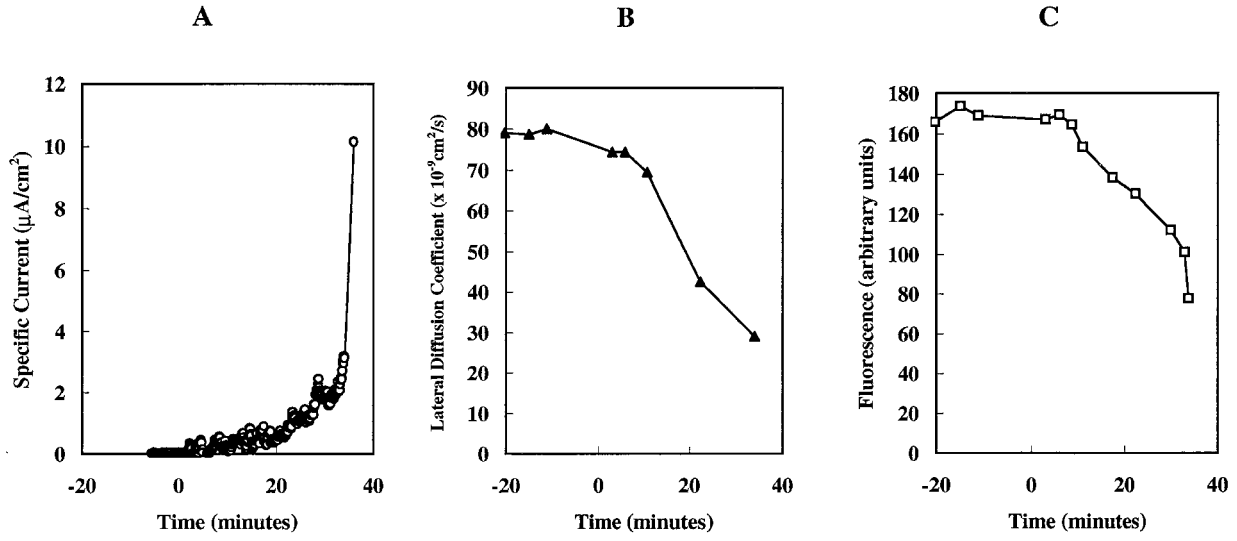

FIGURE 5 Simultaneous measurement of conductivity (*A*, ○), lateral diffusion (*B*, ▲), and fluorescence (*C*, □). PLBs (POPC:DOPE:CHOL, 7:3:10) containing 1 mol% NBD-Chol were formed and the initial fluorescence, lateral diffusion of the probe, and the conductivity of the PLB were determined with time. Avenacin A-1 (1.0 μM final concentration) was then added to the *cis* side (time 0 min), and the same three parameters were again measured with time.

that digitonin has no absolute requirement for sterol in the membrane to cause disruption (Gogelein and Huby, 1984). The reason for the difference in the behavior of digitonin in PLBs and liposomal membranes is not clear, but could be due to the method of PLB formation. The method used by Gogelein and Huby (1984) involved painting PLBs from a solution of decane, a technique known to produce PLBs with a relatively high solvent content. This could interfere with the measurement. To reduce the amount of solvent in the PLBs, we have used the Montal-Mueller (Montal and Mueller, 1972) method to form PLBs. This method of PLB formation results in bilayers with a high specific capacitance and which are therefore considered to be virtually solvent-free (Ladha et al., 1996). The carbohydrate moiety plays a vital role in the mechanism of sterol-avenacin interaction. The monodeglucosyl, bis-deglucosyl, and aglycone derivatives of the avenacin A-1 do not disrupt membranes, but according to step one the aglycone portion of the saponin should insert into the bilayer and then bind to sterols. However, with PLBs containing 50 mol% cholesterol the lateral diffusion coefficient of the sterol probe failed to be influenced by the derivatives of avenacin A-1. This suggests that although the derivatives of avenacin A-1 are inserting into the membrane, the intact carbohydrate unit is required for the reorganization of membrane sterols, resulting in a reduction in the lateral diffusion coefficient.

Step three involves the formation of the transmembrane pore (Fig. 8 *C*). As expected, avenacin A-1, like other saponins (Nishikawa et al., 1984; Gogelein and Huby, 1984; Johnson et al., 1986), causes an increase in the permeability of the membrane. The macroscopic current-voltage relationship showed a voltage-independent behavior. Results similar to these have been obtained for a saponin mixture (Gogelein and Huby, 1984). This indicates that the addition of avenacin A-1 to the *cis* side of the PLB induces rearrangements of the membrane lipids to form a transmem-

TABLE 1 Effect of 1.0 μM avenacin A-1 or the deglucosyl derivatives on the lateral diffusion coefficient of the NBD-Chol probe in PLBs

Addition	Bilayer composition	Lateral diffusion ($\times 10^{-9}$ cm^2 s^{-1})		Molar ratio Avenacin:Chol
		Before addition*	After addition#	
Avenacin A-1	POPC:DOPE:Chol (7:3:1.1)	98.06 ± 6.24	82.36 ± 9.43§	1:2.5
Avenacin A-1	POPC:DOPE:Chol (7:3:5.4)	80.91 ± 7.50	14.31 ± 4.28§	1:9
Avenacin A-1	POPC:DOPE:Chol (7:3:10)	77.29 ± 4.29	31.34 ± 4.35§	1:13
Monodeglucosyl	POPC:DOPE:Chol (7:3:10)	78.44 ± 5.40	74.65 ± 4.52	1:13
bis-Deglucosyl	POPC:DOPE:Chol (7:3:10)	68.71 ± 4.52	62.14 ± 5.03	1:13
Aglycone	POPC:DOPE:Chol (7:3:10)	68.50 ± 5.72	65.69 ± 6.14	1:13

*Control values of lateral diffusion for the probe before the addition of the avenacin A-1 or its derivatives.

#Steady-state values of lateral diffusion for the probe after exposure of the PLB to avenacin A-1, for a time period that caused significant permeabilisation (typical exposure time of 40 min) or after exposure to the derivatives for an equivalent time period. Data values represent mean ± SD of three different experiments.

§Data values that are significantly different from control values ($p < 0.05$).

TABLE 2 Effect of 1.0 μM avenacin A-1 on the lateral diffusion coefficient of the NBD-PE probe in PLBs

Bilayer composition	Lateral diffusion ($\times 10^{-9}$ cm^2 s^{-1}) Before addition*	After addition#	Molar ratio Avenacin: Chol
POPC:DOPE(7:3)	127.19 ± 5.40	122.96 ± 5.44	—
POPC:DOPE:Chol(7:3:1.1)	91.13 ± 5.58	77.49 ± 6.96§	1:2.5
POPC:DOPE:Chol(7:3:5.4)	43.46 ± 3.02	30.78 ± 1.74§	1:9
POPC:DOPE:Chol(7:3:10)	57.86 ± 2.56	41.17 ± 2.16§	1:13

*Control values of lateral diffusion for the probe before the addition of the avenacin A-1 or its derivatives.

#Steady-state values of lateral diffusion for the probe after exposure of the PLB to avenacin A-1, for a time period that caused significant permeabilization (typical exposure time of 40 min) or after exposure of PLBs without cholesterol for an equivalent time period. Data values represent mean ± SD of three different experiments.

§Data values that are significantly different from control values ($p < 0.05$).

brane pore. The mechanism by which this occurs is not yet clear, but we speculate that after binding the sterol molecules, the sugar residues of the avenacin A-1 interact and cause the aggregation of the sterol-avenacin A-1 complex.

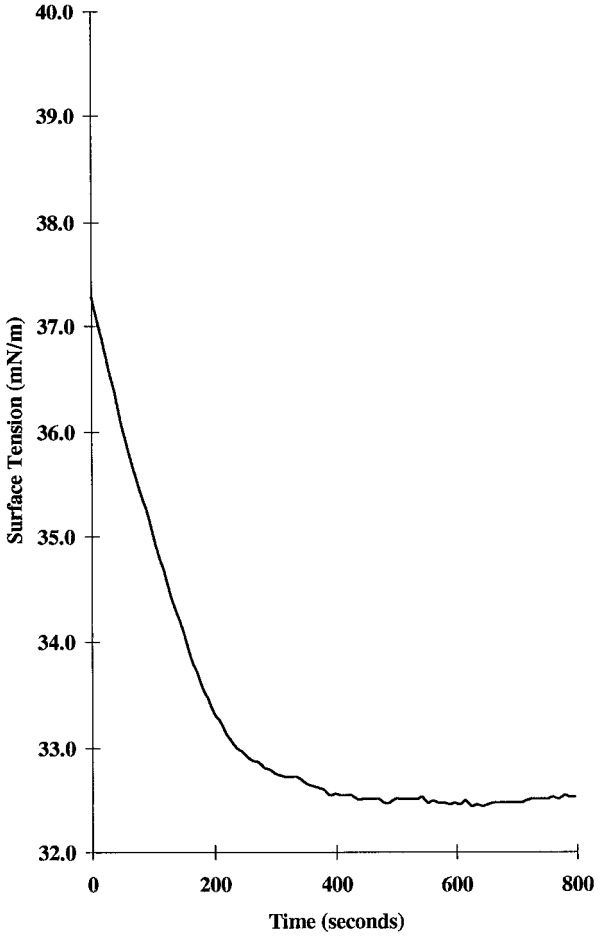

FIGURE 6 The change in the surface tension of cholesterol-free phospholipid monolayers (POPC:DOPE, 7:3) after the addition of avenacin A-1 to the subphase.

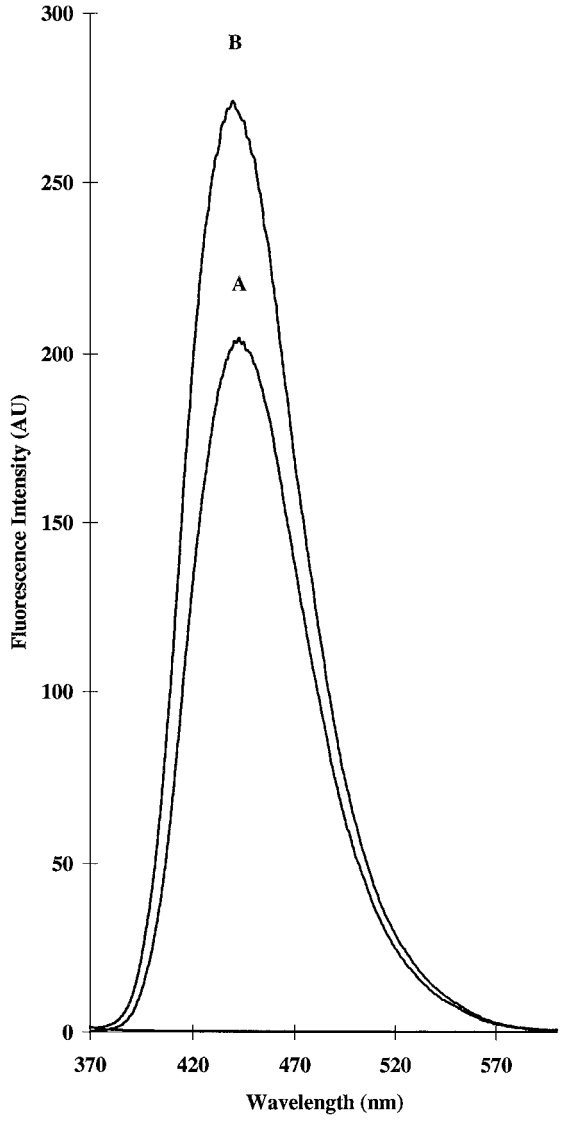

FIGURE 7 The fluorescence emission spectra of avenacin A-1 in the absence (*A*) and presence (*B*) of cholesterol-free liposomes (POPC:DOPE, 7:3).

This may lead to the rearrangement of the bilayer lipids and subsequent formation of the pore. The reorganization of the membrane by other saponins has been visualized by transmission electron microscopy, which indicates the formation of an array of permanent ring-shaped structures (Bangham and Horne, 1962; Seeman, 1974). Using the FRAP method, Ishida et al. (1993) did demonstrate that digitonin reduced the diffusion coefficient of a lipid probe DiI in cultured cardiac cells. We have shown a similar reduction in lateral diffusion of the phospholipid probe in cholesterol-containing PLBs exposed to avenacin A-1. The magnitude of the decrease was of the same order as that found for the decrease in diffusion upon insertion of protein into the artificial membranes, suggesting that lateral movement of phospholipids is impeded by an array of structures formed within the membrane (Ishida et al., 1993). The lateral diffusion of

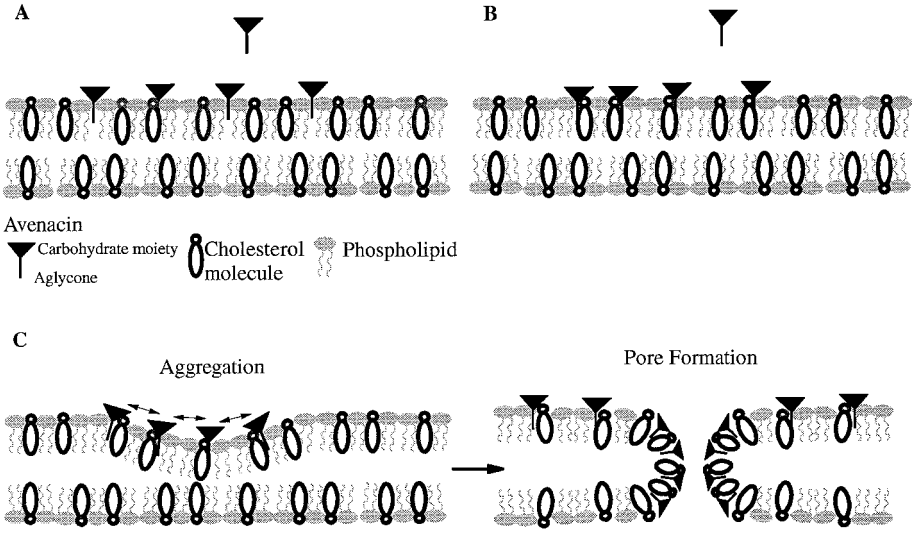

FIGURE 8 The proposed model for the pore formation by avenacin A-1.

the sterol probe was reduced by a larger magnitude than that of the phospholipid probe in cholesterol-containing PLBs exposed to avenacin A-1. This observation suggests that the sterol forms part of a complex array of structures that diffuse slowly within the plane of the membrane. It should be noted that no immobile domains were detected by the FRAP method. This shows that although the membrane sterol formed a more slowly diffusing complex with avenacin A-1, it was not immobilized within the plane of the membrane. A ratio of one avenacin A-1 molecule to nine sterols produced a maximum reduction in lateral diffusion. However, the stochiometry of sterol per pore is not easy to establish using the FRAP data. The reason for this lies in the fact that from the steady-state conductance it is clear that only a small fraction (1%) of the bilayer area is covered by pores. This alone is not sufficient to account for the 80% reduction in lateral diffusion of the sterol probe. It is probable that the large proportion of the reduction in lateral diffusion is due to avenacin A-1 complexing with sterol in the membrane without forming pores. This may result in an array of avenacin A-1/sterol structures varying in size and represent intermediate structures of the fully formed pore.

Removing only one sugar residue results in a complete loss of the pore-forming ability of avenacin A-1. Similar results have been shown for the glycoalkaloid α-tomatine (Keukens et al., 1996). These results contrast with the study of Nishikawa et al. (1984), who showed that removal of one sugar residue from the saponin digitonin did not result in a loss of activity. Removal of subsequent sugar residues resulted in progressive loss of activity. As pointed out by Keukens et al. (1996), removal of three sugar residues from digitonin to give glucosyl-galactosyl-digitogenin produces a sugar structure identical to that of α-tomatine with the xylose and glucose removed (γ-tomatine). However, the glucosyl-galactosyl-digitogenin retains 50% of its membrane-disrupting activity, whereas γ-tomatine is inactive. The only difference between the two molecules is that the aglycone structure of digitonin contains extra hydroxyl groups in the first and fourth rings. This shows that the carbohydrate residues are important in determining the membrane-disrupting activity of these compounds but are not the only factor.

The decrease in fluorescence of the NBD-cholesterol in PLBs exposed to avenacin A-1 shows that there are two events that may be taking place. First, NBD-labeled lipids are weakly fluorescent in an aqueous environment but highly fluorescent in organic solvents (Chattopadhyay, 1990), which could mean that the reduction in fluorescence may be due to the NBD group being transferred to a more hydrophilic membrane environment when exposed to avenacin A-1. This may be the interior of the avenacin-sterol pore structure in the membrane, which will be in contact with the aqueous buffer. Second, an increase in the local concentration of the probe in the membrane results in self-quenching (Hoekstra, 1982). The suggestion is that the decrease in fluorescence could be due to self-quenching of the NBD group when it is sequestered in the avenacin-sterol structures.

In conclusion, the mechanism of avenacin-induced membrane disruption involves the reorganization of the membrane cholesterol into pores. An intact sugar moiety on the avenacin A-1 is essential for this process. The avenacin-cholesterol pore structure shows an ohmic current-voltage relationship, and avenacin A-1-sterol complexes diffuse more slowly in the plane of the bilayer than free membrane cholesterol.

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