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The effect of sterols on the sensitivity of membranes to the channel-forming antifungal antibiotic, syringomycin E

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Abstract

The ability of three sterols of different structure to influence the interaction of syringomycin E (an antifungal antibiotic that forms voltage dependent channels in planar lipid bilayers) with a planar lipid bilayer was evaluated. The rate of increase of bilayer conductance induced by syringomycin E was about 1000-times less in bilayers containing 50 mol% of cholesterol compared to bilayers without sterols. The effect of ergosterol (the primary sterol of fungal cells) on the sensitivity of bilayers to syringomycin E was much weaker than that of cholesterol, while stigmasterol (one of the main sterols of plant cells) did not significantly influence the ability of syringomycin E to induce a conductance increase in the bilayer. None of the sterols altered the single channel conductance properties of syringomycin E. These observations suggest that cholesterol affects the sensitivity of target membranes to syringomycin E by enlarging the energy barrier for channel formation rather than participating in channel formation itself.

Keywords: Syringomycin E; Ion channel; Sterols; Lipid bilayer

1. Introduction

Syringomycin E and its analogues are antifungal cyclic lipodepsipeptides produced by certain strains of the bacterium *Pseudomonas syringae* pv. *syringae* [1,2]. Physiological and biochemical studies have shown that syringomycin E (Fig. 1) acts on the fungal plasma membrane altering several of its functions, including H^+, Ca^{2+}, and K^+ transport, protein phosphorylation, and H^+-ATPase activity [1–4]. It was reported that syringotoxin, a related cyclic lipodepsipeptide, forms anion selective, voltage-sensitive channels in planar lipid bilayers [5,6]. Hutchison et al. [7] reported that syringomycin E induced single channel activity in planar lipid bilayers. We further observed [8] that syringomycin E induces channels that, similar to those of colicin E1 [9], are opened by positive voltages and closed by negative voltages.

Experiments with yeast cells demonstrate that, similar to the polyene class of antifungal drugs, the antifungal activity of syringomycin E is dependent upon the presence of certain sterols in the plasma membrane of the fungal cells [10,11]. Syringomycin

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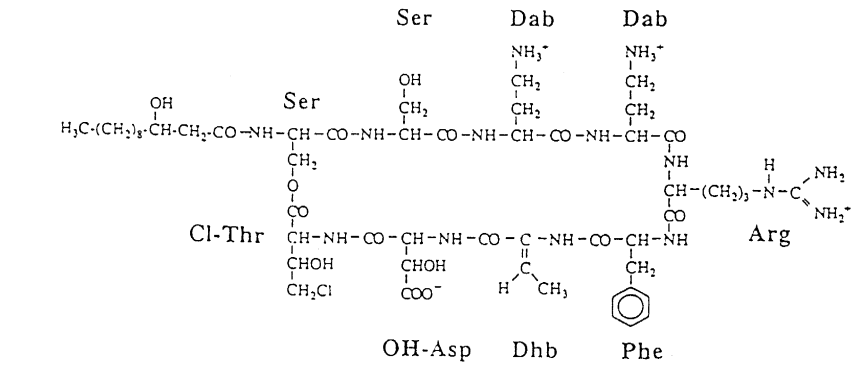
E-resistant mutants of *Saccharomyces cerevisiae* were analyzed to determine the number and functions of genes necessary for growth inhibition by syringomycin E [10]. Among eight genetic classes of mutants, five were also resistant to the polyene antibiotic, nystatin. Since nystatin binds to ergosterol (a sterol found specifically in fungal plasma membranes), it was speculated that the mutants had alterations in ergosterol levels. Further analysis showed that the mutants were indeed deficient in ergosterol in comparison with the parent strain [10,12], suggesting that sterols are required for syringomycin E action. The possible role(s) of sterols in the interaction of syringomycin with the target membranes remained unclear.

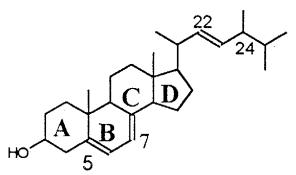
While studying the mechanism of membrane activity of syringomycin with the planar lipid bilayer technique, we observed that the antibiotic forms ion channels in bilayers containing no sterols [8], although a possible role for sterols as modulators of membrane sensitivity to syringomycin remained unstudied. Here we present the results of our studies on the influence of sterols of different structure on the sensitivity of planar bilayers to syringomycin E as well as on the properties of single channels induced by syringomycin E.

2. Materials and methods

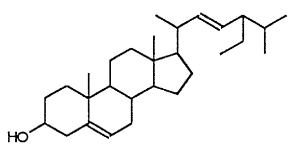
Synthetic 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidyl-



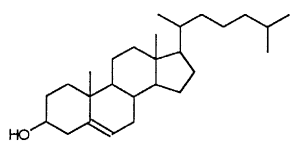
Syringomycin E



Ergosterol



Stigmasterol



Cholesterol

Fig. 1. Structures of syringomycin E (zwitterionic form) (from Fukuchi et al. [41]) and the sterols, cholesterol, ergosterol, and stigmasterol employed in this report. Abbreviations: Arg, arginine; OH-Asp, 3-hydroxyaspartic acid; Dab, 2,4-diaminobutyric acid; Dhb, dehydro-2-aminobutyric acid; Cl-Thr, 4-chlorothreonine; Ser, serine; Phe, phenylalanine.

ethanolamine (DOPE) were purchased from Avanti Polar Lipids, Pelham, AL. The sterols, cholesterol, ergosterol and stigmasterol, were purchased from Sigma Chemical Co., St. Louis, MO. All electrolytes were reagent grade. Water was doubly distilled and deionized. Salt solutions for bilayer experiments were 100 mM NaCl buffered by 5 mM MOPS to pH 6.0. Syringomycin E was purified to homogeneity as described previously [13].

Virtually solvent-free bilayers were prepared as described by Montal and Mueller [14]. Two symmetrical halves of a Teflon chamber with solution volumes of 1 cm^3 were divided by a 15-μm-thick Teflon partition containing a round aperture of about 30 μm diameter. Hexadecane in n-hexane (1:10, v/v) was used for aperture pretreatment. 'Virtual ground' was maintained at the *trans* side of the bilayer. Hence positive voltages mean that the *cis* side compartment is positive with respect to the *trans* side. Positive currents are therefore those of cations flowing from *cis* to *trans*. All experiments were performed at room temperature. A detailed description of methods used for bilayer preparation and single channel data analysis may be found elsewhere [15,16].

Syringomycin E was added to the aqueous phase at one (*cis*) side of the bilayer from stock solutions (1 mg/ml) in water.

2.1. Sensitivity of bilayers of varying sterol composition to syringomycin E

The common approach to estimating the sensitivity of membranes to channel-forming agents is to measure the dependence of bilayer steady-state conductance on the concentration of the channel-former in the bath solution. However, with syringomycin, measuring the dependence of bilayer steady state conductance on the concentration [8] with a constant voltage across the bilayer was very difficult, because a steady state can be reached only across a narrow range of low voltages. It was necessary, therefore, to choose an alternative approach to measuring the sensitivity of bilayers to syringomycin. This alternative technique makes use of the dependence of turn-on rate of the conductance increase (instead of steady-state conductance) on the applied positive voltage at a constant concentration of syringomycin E (3 μg/ml) in the *cis* compartment of the bath solution.

2.2. Voltage dependence of turn-on rates

The turn-on and turn-off macroscopic current kinetics following the application of positive and negative voltage pulses, respectively, to the bilayers doped with one side addition of channel-former are very similar for syringomycin E [8] and for colicin E1 [9]. Based on the logic developed by Abrams et al. [9], we used the voltage dependence of turn-on rates to compare the effect of a given concentration of syringomycin E in bilayers containing sterols of varying structure. We assume that gating of the syringomycin E channel is a two-state process (switching between O (*open*) and C (*closed*) states),

$$C \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} O$$

and all channels are initially in the closed state, i.e., $[\mathbf{o}]_{t=0} = 0$. Then initially

$$d[\mathbf{o}]/dt = k_1[\mathbf{c}]$$

or

$$dg/dt = (g_{\text{single channel}})d[\mathbf{o}]/dt = (g_{\text{single channel}})k_1[\mathbf{c}]$$

where g is the syringomycin-induced conductance. Therefore, if the total number of channels (open plus closed) in the membrane remains constant, and all of the channels are initially closed, dg/dt will be linear and proportional to k_1 at short times after turn-on pulses are applied. In experiments designed to measure the voltage dependence of dg/dt, positive voltage pulses of sufficient amplitude were applied to insure that subsequent linear increases in conductance for short times represented only a small fraction of the total possible bilayer conductance. Voltage was alternated between positive and subsequent negative pulses from 40 mV to 200 mV in steps of 20 mV. For example, first +40 mV is applied. If there is no effect, the voltage is turned off, and then +60 mV is applied. If, now, the current grows, after 10–60 s, the voltage is switched to −60 mV. After the conductance comes to zero, +80 mV is applied, and so on. Prior to each successive positive pulse, the negative one was applied to bring the conductance to zero.

Initial turn-on rates were determined from the slope $[(dI/dt)_{t=0}$ of the I vs. t line at short times at the beginning of the growth of the current, after this process became linear (see Fig. 2C). $(dI/dt)_{t=0}$ was converted to $(dg/dt)_{t=0}$ and plotted semi-logarithmically as a function of voltage. The $\ln(dg/dt)_{t=0}$ vs. V was linear, and its slope was fitted using a non-linear least-squares fitting program (Origin 4.0, Microcal Software, MA, USA).

For each applied voltage the average value of $(dI/dt)_{t=0}$ was estimated using 3–5 membranes.

3. Results

3.1. The macroscopic conductance of bilayers modified with syringomycin E

We demonstrated earlier that the addition of syringomycin E to the bath solution at one side of a bilayer induced an increase in bilayer conductance in a voltage-dependent manner [8]. The formation of channels in bilayers made from DOPS doped with syringomycin E at one side (1–40 μg/ml) was

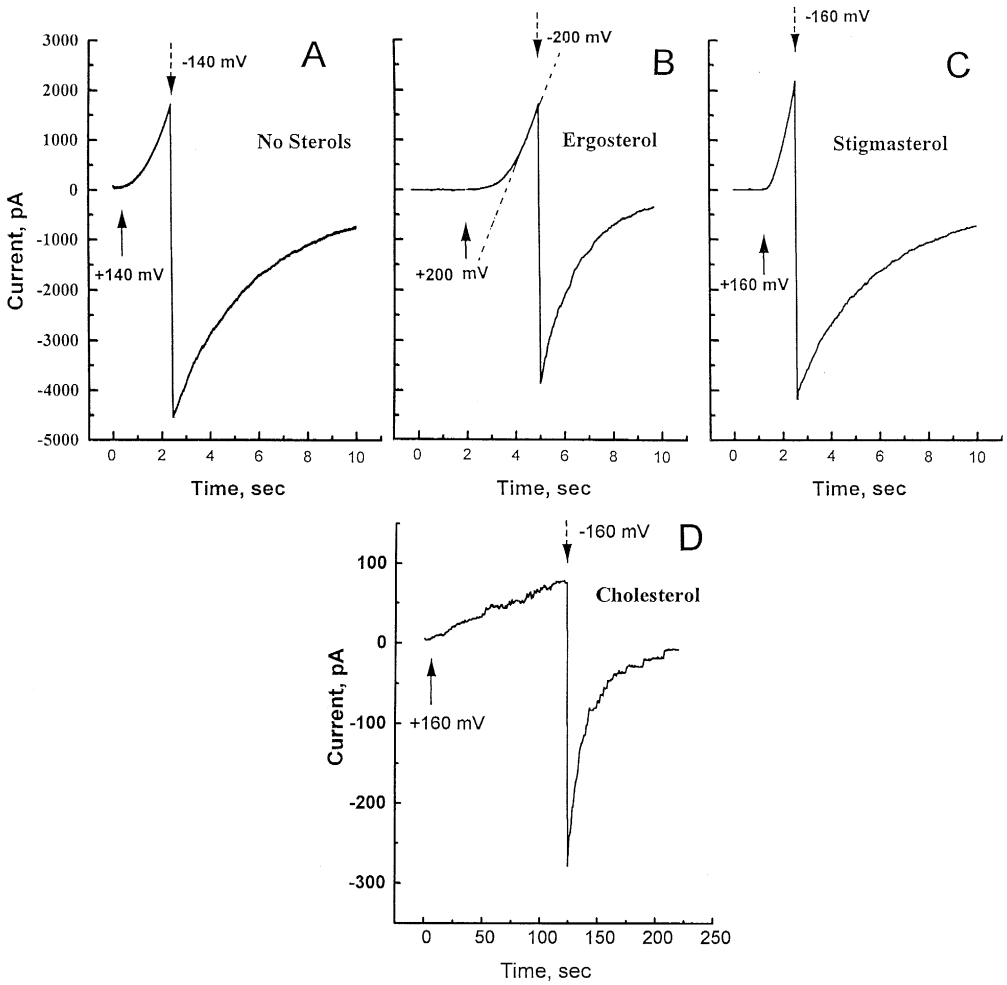


Fig. 2. Time course of the bilayer current in field-reversal experiments in the presence of syringomycin E with the bilayers of different sterol composition – (A) DOPE/DOPS = 1:1 (no sterols); (B) DOPE/DOPS/ergosterol = 1:1:2 (mole); (C) DOPE/DOPS/stigmasterol = 1:1:2; and (D) DOPE/DOPS/cholesterol = 1:1:2. The application of positive voltage (marked by the solid arrows) was followed by a step-like change to negative voltage of the same amplitude (marked by the dotted arrows). The concentration of syringomycin E (*cis* side only) in the bathing solution (100 mM NaCl, 5 mM MOPS (pH 6.0)) was 3 μg/ml. Syringomycin E was added to bath 10–20 min before the records were taken. An example of fitting of the slope of the linear part of the beginning of the current growth after the application of positive voltage is shown in panel B. The dotted line is the theoretical curve.

greatly affected by *cis*-positive voltage. A change of voltage from a positive to a negative value resulted in (1) an abrupt increase in the single channel conductance (the rate of increase was voltage-dependent), simultaneous with (2) a closing of these channels and an exponential decrease in macroscopic conductance over time [8].

Examples of the time course of current across a bilayer formed from the equimolar mixture of DOPS and DOPE with different sterols (50 mol%) modified by one-sided addition of syringomycin E in response to, first, a positive voltage step, and then, a negative one are shown in Fig. 2. It is clear from the data depicted in Fig. 2 that addition of any of the three sterols to the bilayer-forming lipid solution does not qualitatively change the effect of field sign: in all cases we observed an increase in the bilayer conductance at positive voltages and the decrease in this conductance at negative voltages. This observation suggests that the presence of sterols does not change the conformation of the syringomycin channels, and thus, does not alter their response to an applied voltage.

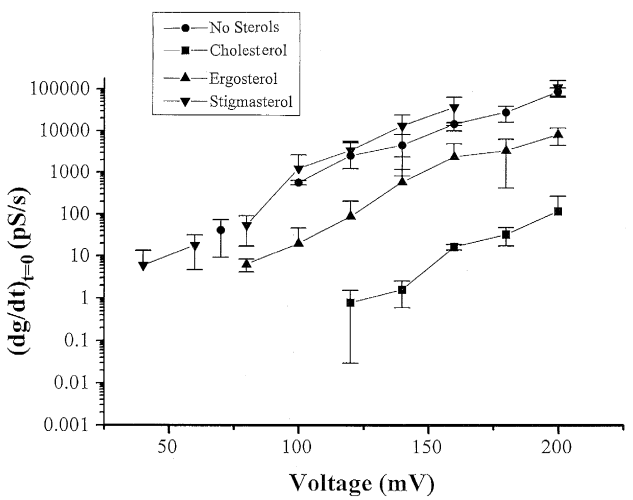


Fig. 3. The curves of dependence of the rate of conductance increase (dg/dt) on the applied positive voltage for bilayers of different sterol composition (DOPE/DOPS = 1:1 with or without 50 mol% of either ergosterol, stigmasterol, cholesterol) are calculated from data similar to those presented in Fig. 2. All rates of current increase were measured at different applied voltages at the same concentration of syringomycin – 3 μg/ml in the bath solution at the *cis* side only. Experimental points are averages from 3–5 bilayers. The error bars represent standard deviations. The calculated slopes of the curves are 17 ± 1.4, 14.9 ± 1.4, 15.9 ± 1.2, and 15.3 ± 1.6 for bilayers containing no sterols, stigmasterol, ergosterol, and cholesterol, respectively.

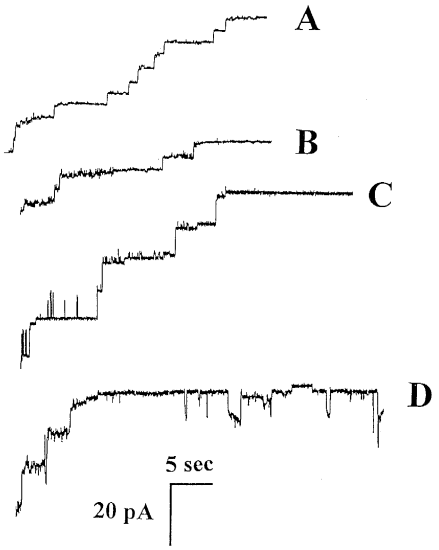


Fig. 4. Records of transmembrane current of bilayers containing a few syringomycin E channels at negative potentials (*cis* side) obtained with cholesterol containing bilayers (DOPE/DOPS/cholesterol = 1:1:2; B, D) and bilayers without sterols (DOPE/DOPS = 1:1; A, C), at −120 mV (A, B) and −180 mV (C, D). The concentration of syringomycin E (*cis* side only) in the bathing solution (100 mM NaCl, 5 mM MOPS (pH 6.0)) was 3 μg/ml. Channel closings and openings are represented by upward and downward deflections, respectively. Data were filtered at 30 Hz, digitized at 1 kHz.

However, the presence of cholesterol greatly decreases the sensitivity of bilayers to syringomycin E. Functions describing the dependence of the rate of conductance increase (dg/dt) to the applied positive voltage for bilayers of different composition (the same concentration of syringomycin E in all cases, 3 μg/ml at *cis* side only) are shown in Fig. 3. These curves were calculated from studies of current kinetics, similar to those presented in Fig. 2. It is clear from the data shown in Fig. 3 that syringomycin E is much less effective at inducing conductance increases in bilayers containing 50 mol% of cholesterol compared with those cases of bilayers containing 50 mol% of ergosterol, or stigmasterol, or in bilayers containing no sterols. The values of the shift of the curves of dg/dt versus voltage (relative to the bilayers without sterols) are +117 mV, +39 mV and 0.4 mV for cholesterol-, ergosterol-, and stigmasterol-containing bilayers, respectively. The addition of

sterols had a minor effect on the slopes, averaging an e-fold increase in the value of dg/dt for every 16 mV. The observed voltage shifts corresponded to 1150- and 10-fold differences in the values of dg/dt at any given voltage for cholesterol- and ergosterol-containing bilayers, respectively, compared with the bilayers without any sterols or bilayers with stigmasterol.

The data demonstrate that (1) the addition of sterols to the bilayer does not change the slope of the dependence of rate of increase of membrane conductance on applied positive voltage; (2) cholesterol strongly diminishes the effect of syringomycin, while the effect of ergosterol on the sensitivity of bilayer to syringomycin is much weaker than that of cholesterol, and stigmasterol is ineffective.

3.2. The properties of single channels formed by syringomycin E in planar lipid bilayers of different sterol composition

Examples of single channel activity observed in planar lipid bilayers formed from the mixture DOPE/DOPS = 1:1 without sterols or from DOPE/DOPS/cholesterol = 1:1:2 (mole) at −120 mV and −180 mV are presented in Fig. 4. There is no difference in the unitary conductance of channels in bilayers of different composition (including bilayers containing ergosterol or stigmasterol (data not shown)). This is true for the range of voltages between −100 to −200 mV (Fig. 5). In all cases the conductance grows from 26 pS at −100 mV to 60 pS at −200 mV (the conductance values refer to the main conductive state), demonstrating that the presence of the 50 mol% of the sterol (either cholesterol, ergosterol, or stigmasterol) does not alter either the channel conductance or its dependence on the applied voltage.

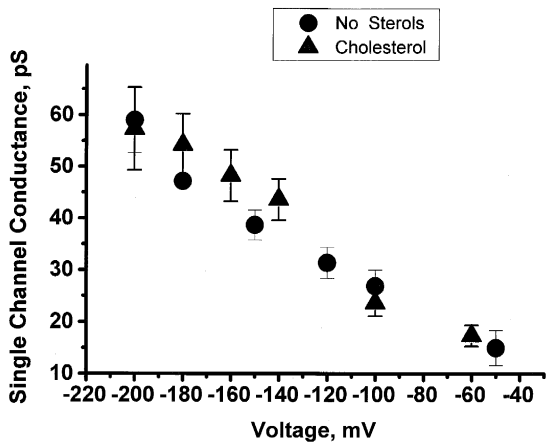


Fig. 5. Voltage dependence of single channel conductances obtained from the records of single channel activity induced by syringomycin E (*cis* side only, 3 μg/ml) in the bathing solution (100 mM NaCl, 5 mM MOPS (pH 6.0)) with cholesterol containing bilayers (DOPE/DOPS/cholesterol = 1:1:2) and bilayers without sterols (DOPE/DOPS = 1:1).

4. Discussion

4.1. The role of sterols in syringomycin activity

The sterols of plasma membranes of eucaryotic cells often play important roles in determining the sensitivities of these membranes to antibiotics and toxins whose activity depends upon increasing membrane permeability. For example, sterols are essential for the cytolytic activity of several large bacterial polypeptide toxins, such as pneumolysin and streptolysin-O [17]. Inclusion of cholesterol in the target membranes strongly potentiates α-toxin effects [18]. The glycoalkaloids (α-solanine, α-chaconine and α-tomatine [19]) and the lipopeptide antibiotic iturin A [20] interact with sterol-containing bilayers, increasing membrane permeability. Sterols are important parts of the actual channels formed by polyene antibiotics [17,21,22], although membranes in the gel state (not common in cells), even without sterols, may be made permeable by polyene antibiotics [23,24]. Yeast mutants resistant to polyene antibiotics usually have lesions in ergosterol biosynthesis, and these mutants accumulate sterols other than ergosterol [25,26].

Recent experiments with yeast cells demonstrate that, similar to the polyene class of antifungal drugs, the antifungal activity of syringomycin E is dependent upon the presence of certain sterols in the plasma membrane of the fungal cells [10,11].

The role of membrane sterols in the interaction of syringomycin E with membranes can be hypothesized to be one of the following.

1. There is a direct interaction with syringomycin E in the formation of the pore structure, i.e., sterols are components of the channel complex.

2. Sterols provide conditions conducive to the assembly and disassembly of channels, but they are not components of channel structure.

To clarify which of these two hypotheses is true, we performed experiments similar to those used previously to clarify the role of lipid components in the formation of channels induced by other agents, including polyene antibiotics, alamethicin and magainin 2 (see below). If sterols directly participate in the formation of syringomycin E-induced channels, it can be expected that their presence and structure will influence the internal structure of channels. Therefore, channel conductance as well as sensitivity of the bilayers to the channel-former will be affected. Such effects have been reported in the ability of sterol structure to influence the conductance of amphotericin B-induced channels [27], the conductance of channels formed by the water-soluble mitochondrial porins [28], the influence of phospholipid structure on the ion selectivity of channels induced by the polypeptide antibiotic, magainin 2 [29], and on sensitivity of lipid bilayers to magainin 2 [30].

If sterols influence membrane sensitivity without directly participating in the formation of syringomycin E channels, it is expected that this influence may result from the effect of sterols on the surrounding environment of the syringomycin E channels and thus, on the partitioning of syringomycin E between the membrane interior and its surface (or electrolyte solution). This effect would likely occur without a change in the channel properties. Such an influence on membrane sensitivity was observed for cholesterol on the voltage-dependent conductance induced by alamethicin [31,32].

The results of the present study suggest that the second hypothesis may more readily explain the effect of cholesterol on the interaction of syringomycin E with lipid bilayers. We demonstrated that cholesterol-containing bilayers are much less sensitive to syringomycin E than are bilayers containing ergosterol, stigmasterol or no sterols at all (Fig. 3). We observed no difference in single channel conductivity or in responses of bilayer current to a change of polarity of transmembrane potential among the bilayers of different sterol composition (Fig. 2 and Fig. 5). Also, the slope of the function, dg/dt versus voltage (Fig. 3), does not change with the modification of the sterol composition of bilayers. This slope is directly related to the value of the effective gating charge, which is equal to the product of the value of electric charge responsible for the channel voltage gating and the distance that this charge passes after the voltage is applied [33]. The unchanged slope, therefore, implies a lack of effect on channel conformation. Taken together, our data suggest that: (i) sterols do not change the properties of the syringomycin E channels in the bilayer and thus, they do not participate directly in syringomycin E channel formation; (ii) the presence of cholesterol in the bilayer increases the energy barrier for the syringomycin channel formation. Therefore, in cholesterol-containing bilayers, higher voltages are necessary to induce a given level of membrane conductance.

4.2. Why does cholesterol protect membranes better than ergosterol and stigmasterol?

The presence of ergosterol influenced bilayer sensitivity to a much lesser extent compared with cholesterol, while stigmasterol was inactive (Fig. 3). These results are consistent with previous observations of protection by exogenously added cholesterol against syringomycin E inhibition of yeast growth [34]. Interestingly, protection was afforded only at times soon after syringomycin E addition and not at later times, suggesting that cholesterol interferes with the initial processes leading to cytotoxicity. Furthermore, ergosterol and stigmasterol afforded some, but significantly less, protection against cytotoxicity.

The question as to why cholesterol is the most active sterol in protecting bilayers against syringomycin E should be addressed. In general, the ability of sterols to influence membrane sensitivity to a drug depends on the structural features of the sterol molecule. These features are more-or-less specific for each drug. For example, the ability of sterols to increase the sensitivity of planar lipid bilayers to amphotericin B correlated with the presence of the 5,7-diene system in the sterol molecule [35–37]. The presence of a double bond or a methyl group in the side chain of the sterol molecule was without effect. The presence of the 5,7-diene system completely blocked the ability of sterols to inactivate gramicidin A channels in planar lipid bilayers [38]. In contrast, the sensitivity of membranes to iturin A and glycoalkaloids was strongly dependent on the structure of

the sterol side chain, but not on the presence of the 5,7-diene system in the sterol nucleus [19,39]. A theoretical analysis revealed a qualitative difference between cholesterol and ergosterol in the distribution of molecular electrostatic potential around the side chain [40].

Our results suggest that branching of the side chain of sterol molecules and/or the presence of the double bond at C_{22}–C_{23} decreases the ability of sterols to protect the membrane against syringomycin E activity. The occurrence of these structural features is the main difference between stigmasterol (with an ethyl group at C_{24} and a double bond at C_{22}–C_{23}) and ergosterol (with a methyl group at C_{24} and a double bond at C_{22}–C_{23}) on the one hand, and cholesterol (with no alkyl substituents at C_{24}, and no double bond at C_{22}–C_{23}) on the other. (The structures of these three sterols are presented in Fig. 1.) The importance of the side chain structure of the sterol is further supported by the fact that the remaining moieties of stigmasterol (with no protective activity) and cholesterol (with high protective activity) are identical.

Acknowledgements

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