Journal of Bioenergetics and Biomembranes, Vol. 37, No. 5, October 2005 (© 2005)
DOI: 10.1007/s10863-005-8645-2

Sphingolipids Influence the Sensitivity of Lipid Bilayers to Fungicide, Syringomycin E

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Received April 13, 2005; accepted June 30, 2005

Sphingolipids with long chain bases hydroxylated at the C4 position are a requisite for the yeast, *Saccharomyces cerevisiae,* to be sensitive to the ion channel forming antifungal agent, syringomycin E (SRE). A mutant *S. cerevisiae* strain, Δ*syr2*, having sphingolipids with a sphingoid base devoid of C4-hydroxylation, is resistant to SRE. To explore the mechanism of this resistance, we investigated the channel forming activity of SRE in lipid bilayers of varying composition. We found that the addition of sphingolipid-rich fraction from Δ*syr2* to the membrane-forming solution (DOPS/DOPE/ergosterol) resulted in lipid bilayers with lower sensitivity to SRE compared with those containing sphingolipid fraction from wild-type *S. cerevisiae*. Other conditions being equal, the rate of increase of bilayer conductance was about 40 times slower, and the number of SRE channels was about 40 times less, with membranes containing Δ*syr2* versus wild-type sphingolipids. Δ*syr2* sphingolipids altered neither SRE single channel conductance nor the gating charge but the ability of SRE channels to open synchronously was diminished. The results suggest that the resistance of the Δ*syr2* mutant to SRE may be partly due to the ability of sphingolipids without the C4 hydroxyl group to decrease the channel forming activity of SRE.

KEY WORDS: Syringomycin E; ion channels; bilayer lipid membranes; sphingolipid; fungicide; *Pseudomonas syringae*.

INTRODUCTION

Syringomycin E (SRE) (Fig. 1A) is a member of a family of cyclic lipodepsipeptides produced by certain strains of the plant bacterium *Pseudomonas syringae* pv. *syringae* (Gross *et al*., 1977; Sinden *et al*., 1971; Takemoto *et al*., 2003). Traditionally regarded as a plant virulence factor, SRE and its analogs are potent fungicides. The exact mechanisms responsible for eliciting the toxic and fungicidal effects of SRE remain unclear, although physiological and biochemical studies have shown that SRE targets primarily the plasma membrane (Takemoto *et al*., 2003). In this regard model studies have shown that SRE forms ion channels in both planar lipid bilayers (Feigin *et al*., 1997, 1996; Gurnev *et al*., 2002; Hutchison *et al*., 1995; Kaulin *et al*., 1998) and red blood cell membranes (Blasko *et al*., 1998). SRE ion channels in model lipid membranes display either "small" or "large" current amplitudes. These two channel types, nevertheless, show identical ion selectivity and polymer exclusion properties, suggesting that they have identical inner radii (Schagina *et al*., 1998; Kaulin *et al*. 1998). Such observations lead to the conclusion that the "large" channels are clusters of the "small" ones exhibiting synchronous opening and closing. The findings support one suggested mechanism of antifungal action of SRE, namely its ability to collapse the membrane potential of susceptible cells.

Experiments with the yeast, *Saccharomyces cerevisiae*, demonstrated that the fungicidal activity of SRE depends upon the presence of specific sphingolipids

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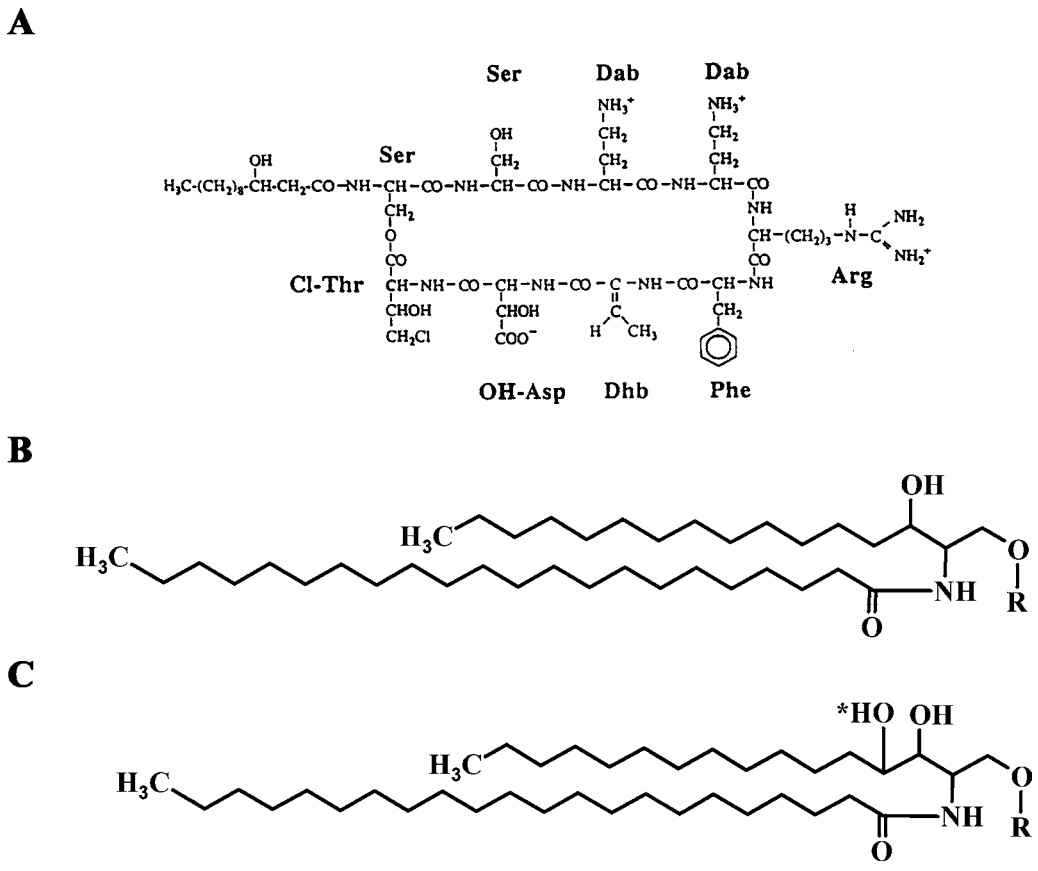

Fig. 1. Structures of SRE (A) and yeast sphingolipids based on dihydroceramide (B) and phytoceramide (C). The zwitterionic form of SRE is shown (with abbreviations: Arg, arginine; OH-Asp, 3-hydroxiaspartic acid; Dab, 2,4-diaminobutyric acid; Dhb, dehydro-2-aminobutyric acid; Cl-Thr, 4-chlorothreonine; Ser, serine; Phe, phenylalanine). Dihydroceramide and phytoceramide-derived sphingolipids are found in Δ*syr2* mutant and wild-type yeast strains, respectively, and are distinguished by a hydroxyl group at C4 position (asterisk) in the latter. R = phosphoinositol or phosphoinositol-mannose, or diphosphoinositol-mannose.

in the plasma membrane of the yeast cells (Grilley *et al.*, 1998; Stock *et al.*, 2000). To show this, SRE-resistant mutants were analyzed to determine the functions of a number of genes found necessary for the ability of SRE to inhibit growth. Among the most prominent of these is the *S. cerevisiae* gene, *SYR2*, required for 4-hydroxylation of sphingoid bases to form phytoceramide in sphingolipid biosynthesis. Mutant strains with defective *SYR2* produce sphingolipids missing the hydroxyl group at the C4 position of the long chain base moiety (dihydroceramide), and these mutants are resistant to SRE. Structures of sphingolipids from mutants and from wild-type cells are presented in Fig. 1B and C. Why sphingolipid hydroxylation confers sensitivity to SRE is unknown. However recent work by Idkowiak-Baldys *et al.* (2004) has shown that C4-hydroxylation influences the physical and structural properties of lipid microdomains suggesting that SRE may interact with lipid rafts and be sensitive to their composition.

Given the results from the yeast sphingolipid biosynthetic mutants, it is likely that sphingolipids affect the membrane sensitivity to SRE. Yet it is not known how sphingolipids influence the sensitivity of membranes to SRE, whether these lipids are receptors for SRE, or whether they might modulate specific parameters of channels formed by SRE.

This paper presents results of studies examining the influence of yeast sphingolipid structure on the sensitivity of planar lipid bilayers to SRE. Our data show that the state of sphingolipid C4 hydroxylation does not drastically change SRE channel properties in lipid bilayers, but that a larger number of SRE channels are formed per unit time with hydroxylated versus non-C4 hydroxylated sphingolipids.

MATERIALS AND METHODS

Chemicals

Synthetic 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids, Inc., Alabaster, AL. Ergosterol was purchased from Sigma, St Louis, MO. All electrolytes were reagent grade. Water was doubly distilled and deionized. The bathing solution for bilayer experiments was 0.1 M NaCl buffered by 5 mM MOPS to pH 6.0. SRE was purified as described previously (Bidwai *et al*., 1987).

Electrical Conductance Measurements on Planar Lipid Bilayers

Virtually solvent-free membranes were prepared as described by Montal and Mueller (1972). 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 100 μm diameter. Hexadecane in *n*-hexane (1:10, v/v) was used for aperture pretreatment. A pair of Ag−AgCl electrodes was used. "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* side.

All experiments were performed at room temperature. A detailed description of methods used for membrane preparation and single channel data analysis may be found elsewhere (Bezrukov and Vodyanoy, 1993; Feigin *et al*., 1996). Syringomycin E was added to the aqueous phase at one (*cis*) side of the bilayer from water stock solutions (1 mg/ml).

Unless otherwise stated, lipid bilayers contained 20 mol% of sphingolipid, where the remaining lipid portion contained DOPS/DOPE/ergosterol in 1:1:1 (M:M:M) ratio.

The channel effective gating charge, Q, was measured in voltage-jump experiments. To determine the effective number of the small channels open at a given membrane potential, the steady-state current was divided by the corresponding current through a single small channel. This procedure gave us the effective number of the open small channels, N_{ch}, which was used to find the effective gating charge, Q. The Q value was obtained from a logarithmic plot of the N_{ch} value as a function of the applied voltage, V, (Hille, 1992; Schagina *et al*., 2003). The Q value was expressed as $d(\ln N_{ch})/d(FV/RT)$, in which F, R and T have their usual meanings of the Faraday and gas constants and the absolute temperature, respectively.

Yeast Sphingolipids

Phosphoinositol-containing sphingolipids were extracted from *S. cerevisiae* strains W303C (MATa *ade2 his3 leu2 trp1 ura3*) and W Δ*SYR2*α (MATa *ade2 his3 leu2 trp1 ura3 syr2::URA3*) as previously described (Grilley *et al*., 1998). Strains W303C and W Δ*SYR2*α synthesize sphingolipids based on C4-hydroxylated phytoceramide and on non C4-hydroxylated dihydroceramide, respectively (Grilley *et al*., 1998). These cell strains are referred to here simply as "wild-type" and "Δ*syr2*," respectively.

One liter cultures were grown in 2-L capacity Fernbach flasks with rotary shaking in yeast extract–peptone–dextrose medium at 28°C for 2 to 3 days. The cells were collected by centrifugation (3000 × *g*, 10 min), followed by two washes with distilled water and repeated centrifugation. Cell pellets were suspended in water to a volume of 2.3 ml/g wet wt. and extracted with an equal volume of 95% ethanol–diethylether–pyridine (15:5:1 vol/vol/vol) at 57°C for 30 min. The extract was centrifuged (3000 × *g*, 10 min) and the supernatant fluid extract removed and adjusted to pH 5.5 with acetic acid. The extract was incubated on ice for 48 h and then centrifuged at 4400 × *g* for 5 min. The pellet was suspended in 1.8 ml petroleum ether, centrifuged at 4400 × *g* for 5 min, and the supernatant fluid collected. An equal volume of absolute ethanol was added to the supernatant and the extract incubated overnight on ice. The extract was then centrifuged at 24, 000 × *g* for 5 min, the pellet suspended in 2 ml methanol:chloroform (1:1 vol:vol), and dried under nitrogen.

The extracts obtained from both wild-type and Δ*syr2* mutant strains were highly enriched in sphingolipids and similar in overall lipid composition to equivalent preparations reported by Smith and Lester (1974). Thin-layer chromatographic analyses and staining with orcinol-H_2SO_4 (Smith and Lester, 1974) showed the presence of mannosyl-diinositolphosphoceramide, mannosyl-inositolphosphoceramide, and inositolphosphoceramide in approximate ratios of 2:1:1, respectively.

RESULTS

Figure 2A and B shows the time course of bilayer currents obtained after the addition of 2 μg/ml SRE to the *cis* side of lipid bilayers containing sphingolipid-rich fractions (hereafter referred to as simply, "sphingolipids") from either the wild-type (Fig. 2A) or Δ*syr2* strain (Fig. 2B). Both lipid conditions (i.e., Fig. 2A and B) show

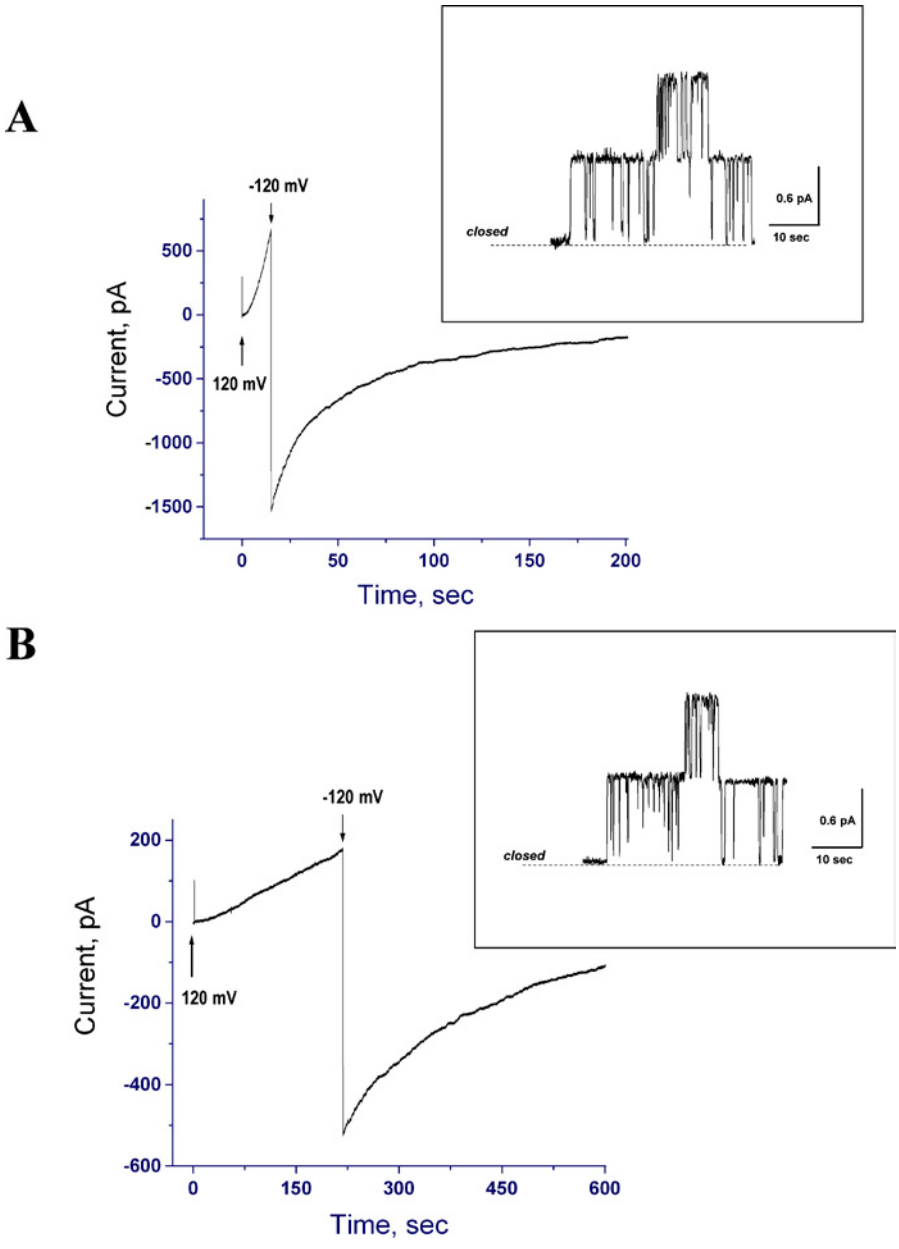

Fig. 2. Time course of bilayer current in field-reversal experiments in the presence of SRE in bilayers composed of either: (A)—DOPS/DOPE/ergosterol = 1:1:1 (M:M:M) with 20 mol% of sphingolipids isolated from wild-type cells; (B)—DOPS/DOPE/ergosterol = 1:1:1 (M:M:M) with 20 mol% of sphingolipid isolated from $\Delta syr2$. The time of voltage application is marked by arrows. The concentration of SRE (*cis* side only) in the bathing solution was 2 μg/ml. Membrane bathing solution was 0.1 M NaCl, pH 6. Transmembrane potential was $\pm$120 mV. Inserts show examples of the current through a bilayer membrane containing up to two SRE ion channels at +150 mV.

qualitatively the same type of current trace. Note that: (i) the current increases with time when the sign of the applied membrane potential is positive, and decreases in absolute value when the sign is negative; (ii) an equal-step change in voltage from positive to negative results in an increase in the absolute value of the transmembrane current. Similar observations have been reported for SRE-induced conductance of bilayers containing more than 20 mol% of DOPS in DOPE bilayers (Feigin *et al*., 1996; Schagina *et al*., 2003). The corresponding small SRE channel traces

are shown in inserts of Fig. 2. There was no difference in unitary current through small SRE channels formed in bilayers of either composition (see also current/voltage (I/V) curves presented in Fig. 5).

The data in Fig. 2 indicate that the presence of any of the two sphingolipids in the membrane-forming solution does not qualitatively affect the field sign and the asymmetric nature of the I/V relationship (also see Fig. 5), but does affect the rate of current change. For bilayers containing sphingolipids from Δ*syr2*, this rate of change of the current (an increase at positive transmembrane potential) is lower than that for bilayers containing sphingolipids from wild-type cells. Also as seen in Fig. 2, at the same SRE concentration and applied potential, more time is required to achieve the same transmembrane current value in the case of bilayers containing sphingolipids from Δ*syr2* (Fig. 2B) than of those containing sphingolipids from wild-type cells (Fig. 2A). For example, 220 s was required for a current of 150 pA to be reached after a transmembrane potential of 120 mV was applied to the bilayer containing sphingolipids from Δ*syr2*, while, in contrast, only 5 s was required for the same level to be reached in bilayers containing sphingolipids from wild-type cells.

The sensitivity of bilayers to SRE was assessed using the approach of Feigin *et al*. (1996, 1997). This analysis determines $(dG/dt)_{t=0}$, the initial rate of change of SRE-induced conductance of the bilayer, which can be thought of as a measure of the channel-forming activity of SRE. Fig. 3 shows the dependence of ln $(dG/dt)_{t=0}$ on the applied voltage (in units of FV/RT) for bilayers of the two sphingolipid types. The data presented in Fig. 3 were obtained from current kinetic curves similar to those presented in Fig. 2. Figure 3 shows that within the voltage range of 100–200 mV, SRE is less effective at inducing current increases in bilayers containing 20% sphingolipids from Δ*syr2* compared with bilayers containing 20% sphingolipids from the wild-type strain. The magnitude of the shift between the two practically parallel lines is 94 mV.

Figure 4 shows the relationship of ln $(dG/dt)_{t=0}$ versus increasing concentrations of sphingolipids from Δ*syr2* (range, 5–30 mol%) with SRE at 1.2 μg/ml and voltage of 80 mV. The value of ln $(dG/dt)_{t=0}$ decreases monotonically with increasing Δ*syr2* sphingolipid concentration. Note that the magnitude of ln $(dG/dt)_{t=0}$ of the single point from bilayers containing 20% wild-type sphingolipid (open circle) is approximately equivalent to that from bilayers containing 5–10% of sphingolipid from the Δ*syr2*.

At low concentrations of SRE in the membrane bathing solution we were able to detect single channels. Examples of these channels are shown in the insert of Fig. 5. Figure 5 displays the current/voltage curves of

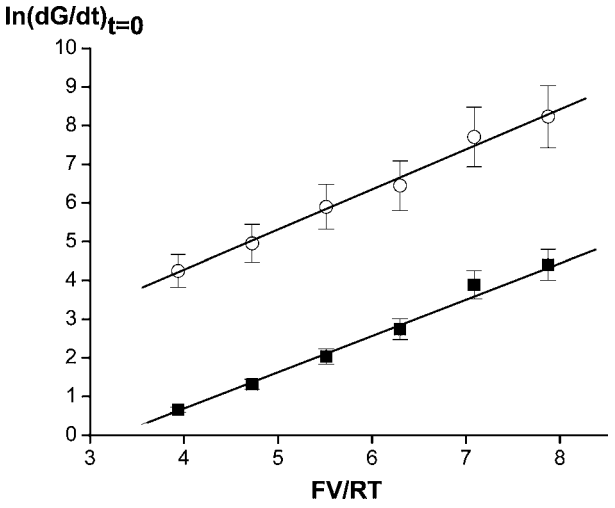

Fig. 3. The dependence of the rate of conductance increase ln $(dG/dt)_{t=0}$ versus applied positive voltage (in units of RT/F, where R is gas constant, F is Faraday's constant, T is absolute temperature) for bilayers composed of either (open symbols), DOPS/DOPE/ergosterol = 1:1:1 (M:M:M) with 20 mol% of sphingolipid from wild-type cells; (solid symbols), DOPS/DOPE/ergosterol = 1:1:1 (M:M:M) with 20 mol% of sphingolipid from Δ*syr2*. The values of $(dG/dt)_{t=0}$ were calculated from data similar to those presented in Fig. 2. (See Feigin *et al*. for details.) Experimental points are averages from three to five bilayers. The error bars represent standard deviations. All experiments were run at 2 μg/ml SRE in the membrane bathing solution at the *cis* side only. Membrane bathing solution was 0.1 M NaCl, pH 6.

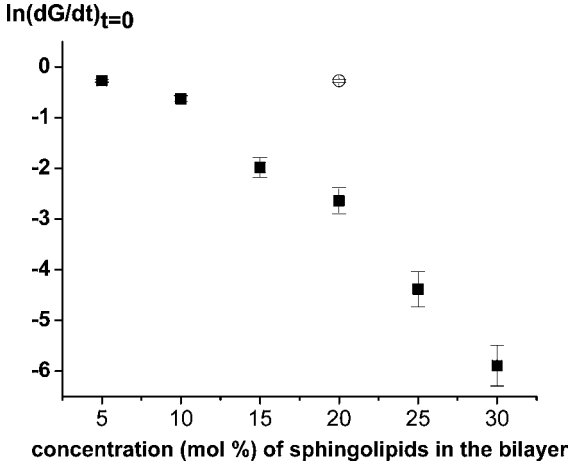

Fig. 4. Bilayer conductance rate change (SRE activity) ln $(dG/dt)_{t=0}$ at 80 mV versus the concentration (from 5 to 30 mol%) of sphingolipid from Δ*syr2* (solid symbols) and at the 20 mol% point of sphingolipid from wild-type cells (open symbols) in DOPS/DOPE/ergosterol = 1:1:1 (M:M:M) bilayers. The error bars represent standard deviations of from three to five bilayers. The concentration of SRE (*cis* side only) in the bathing solution was 1.2 μg/ml. Membrane bathing solution was 0.1 M NaCl, pH 6.

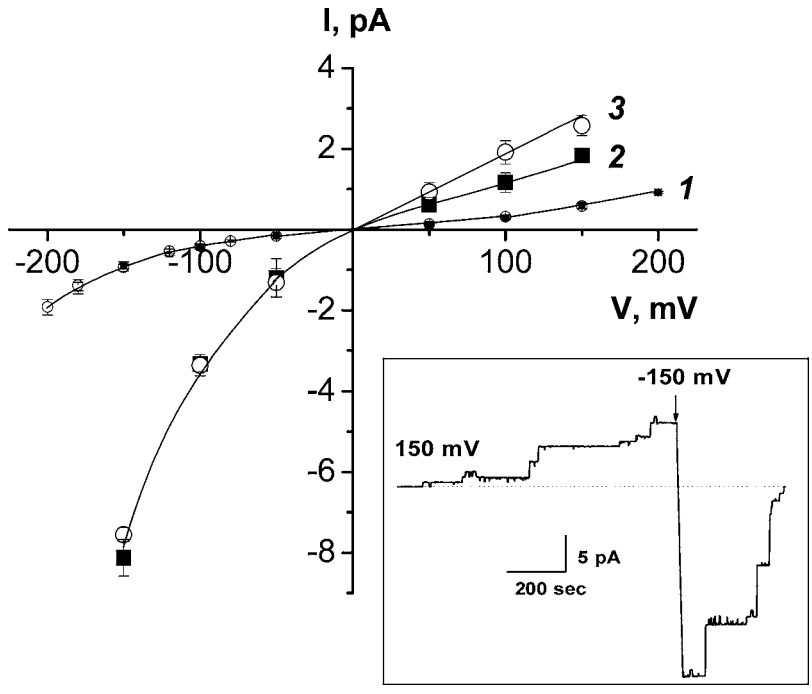

Fig. 5. Current/voltage (*I/V*) curves for small (*curve 1*, small symbols) and large (*curves 2* and *3*, large symbols) SRE channels in bilayers composed of DOPS/DOPE/ergosterol = 1:1:1 (M:M:M) with 20 mol% sphingolipids from wild-type cells (*open symbols*); DOPS/DOPE/ergosterol = 1:1:1 (M:M:M) with 20 mol% sphingolipid from Δ*syr2* (*solid symbols*). Membrane bathing solution was 0.1 M NaCl, pH 6. The two *I/V* curves for large SRE channels (*curve 2 and 3*) superimpose at negative membrane voltages and two *I/V* curves (*curve 1*) for small channels superimpose at all range of membrane voltages. Insert shows current fluctuations corresponding to opening and closure of elementary SRE-channels and clusters. Application of −150 mV indicated by an arrow.

small (*curve 1*) and large (*curves 2 and 3*) SRE channels in membranes containing either 20 mol% sphingolipids from Δ*syr2* (solid symbols) or 20 mol% sphingolipids from wild-type cells (open symbols). These curves demonstrated pronounced superlinear dependence of single unit currents at negative *V*. *I/V* curves for small channels were indistinguishable for bilayers of both membrane compositions within a voltage range of −200 mV to +200 mV. However unitary currents for large conductance states at positive voltages were ∼1.5 times larger in case of wild-type sphingolipid containing membranes compared with those in bilayers containing sphingolipis from Δ*syr2*.

We also calculated the relative number of large SRE channels, *S*, as $N/(N+n)$, where *N* is the number of large channels and *n* is the number of small channels from traces similar to those presented in the insert of Fig. 5. It was observed that the relative number of large channels was almost twice as large in bilayers containing wild-type sphingolipids than in bilayers containing sphingolipis from Δ*syr2* (0.31 ± 0.12 vs. 0.17 ± 0.08, respectively, average of 44 experiments) and *S* was not affected by the value of the membrane potential (in a range of −200 mV to +200 mV). Accordingly, the contribution of large channels to the total transmembrane current [defined as $I_l/(I_l + I_s)$, where I_l and I_s are currents passing through all large and small channels, respectively] was also diminished in Δ*syr2* sphingolipid containing membrane. $I_l/(I_l + I_s)$ equaled 0.8 ± 0.1 and 0.5 ± 0.1 (average of 37 experiments) for the SRE-modified membranes containing wild-type and Δ*syr2* sphingolipids, respectively.

Figure 6 presents results of a voltage-jump experiment (insert) and the corresponding dependence of the effective number (see Materials and Methods section) of small SRE channels under steady-state conditions on membrane voltage for bilayers containing either wild-type or Δ*syr2* sphingolipids. The values of the gating charge, *Q*, obtained from this dependence as described in Materials and Methods section, were found to be 1.0 ± 0.1 for bilayers containing wild-type or Δ*syr2* sphingolipid. These data indicate that the gating charge of the SRE channels is the same regardless of the type of sphingolipid incorporated into the membrane. Figure 6 also shows

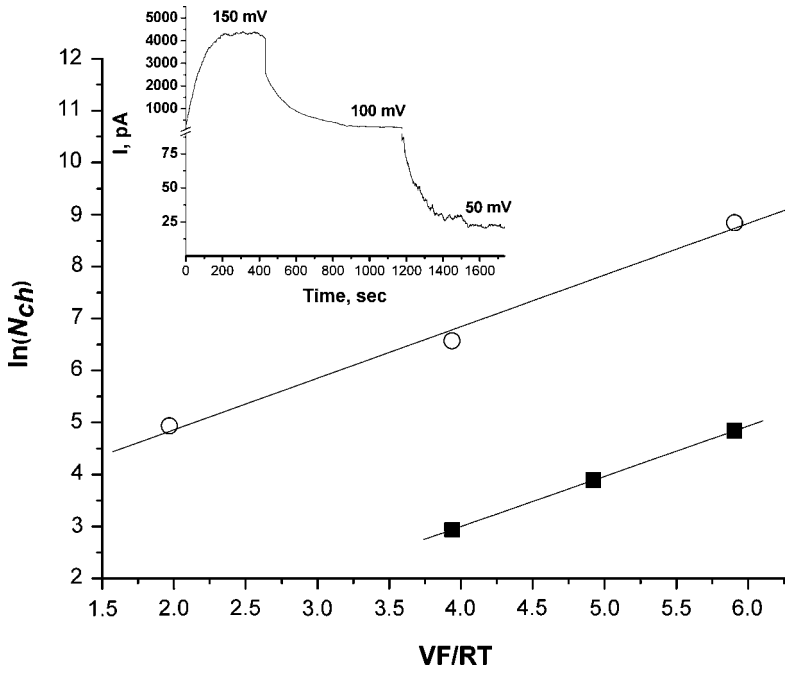

Fig. 6. A semilogarithmic plot of the number of the open SRE channels at steady-state conditions as a function of the applied voltage for bilayers containing sphingolipids. The number of channels at steady-state conditions was obtained from the steady-state levels of the current (see insert). The insert gives the time dependence of the current through the SRE modified membrane containing sphingolipids from the wild-type cells ($C_{SRE} = 1$ μg/ml, *cis* side only) in response to successive changes in amplitude of applied voltage: From 0 to 150 mV, from 150 to 100 mV, and from 100 to 50 mV. See Materials and Methods section for further information. Lipid bilayers were formed from DOPS/DOPE/ergosterol = 1:1:1 (M:M:M) with 20 mol% sphingolipid from wild-type cells (open symbols), and with 20% sphingolipid fromΔ*syr2* (solid symbols). Membrane-bathing solution was 0.1 M NaCl at pH 6.

that the effective number of open small channels under steady-state conditions at the same SRE concentration and the transmembrane potential value was 40 times larger in membranes containing sphingolipids from the wild-type compared with those in membranes containing sphingolipids from Δ*syr2*.

DISCUSSION

In addition to their role as membrane structural components, sphingolipids are involved in mediating cell–cell interactions, anchoring membrane proteins (Hannun and Bell, 1989), and in stimulating the release of Ca^{2+} from intracellular stores (Kindman *et al*., 1994). They also serve as receptors for many ligands, including bacterial enterotoxins (Fishman *et al*., 1993), earthworm hemolysin (Lange *et al*., 1997) and *Escherichia coli* verotoxin (Lingwood, 1993). In yeast, sphingolipids play central roles in resistance to high temperatures, low pH, and osmotic stress (Dickson *et al*., 1997; Jenkins *et al*., 1997; Patton *et al*., 1992). To our knowledge the question concerning the effect of sphingolipids on ion-channel formation in lipid bilayers has not been addressed.

In yeast, specific structural features of sphingolipids influence the antifungal efficacy of SRE (Stock *et al*., 2000). Data in this current report compare the SRE sensitivity of lipid bilayers containing a sphingolipid-rich fraction from wild-type yeast strain (with C4 hydroxylated ceramide) with those that contain Δ*syr2* sphingolipids (with non-C4 hydroxylated ceramide) (Fig. 1B and C). The sphingolipid concentration (20 mol%) in the present bilayer experiments is comparable to that found in the yeast plasma membrane (Patton and Lester, 1991).

The main result shows that SRE channel formation occurred more readily, and the effective number of small channels under steady-state conditions was ~40 times larger, in bilayers containing sphingolipids from the wild-type cells compared with those bilayers containing Δ*syr2* sphingolipids.

Comparing the activity of SRE in bilayers containing Δ*syr2* sphingolipids with bilayers containing wild-type

sphingolipids, we demonstrated that:

1. More time is required to reach the same current value for bilayers containing Δ*syr2* sphingolipids compared with those containing sphingolipids from wild-type cells at the same applied voltage and SRE concentration (Fig. 2A and B). Taking into consideration that the conductance of small SRE channels formed in bilayers with either sphingolipid composition does not differ (Fig. 5 and inserts in Fig. 2), one can conclude that the effective number of small SRE channels being formed (in a unit of time) in bilayers containing C4 hydroxylated sphingolipids is greater than that in bilayers containing non-C4 hydroxylated sphingolipids.
2. At equivalent SRE concentrations, the same value of $(dG/dt)_{t=0}$ can be achieved at about a 100 mV higher applied potential (Fig. 3) in bilayers containing Δ*syr2* sphingolipids versus bilayers with wild-type sphingolipids. In a range of applied voltages of 100–200 mV, the value $(dG/dt)_{t=0}$ is about 40 times larger for bilayers containing sphingolipids from wild-type cells than from the mutant type (Fig. 3). In addition, under steady-state conditions, a 40-fold difference in effective number of small SRE channels was observed in bilayers containing wild-type sphingolipids compared with bilayers containing Δ*syr2* sphingolipids (Fig. 6). Interestingly, we noticed that membranes containing Δ*syr2* sphingolipids required a relatively high concentration of SRE in the membrane bathing solution (0.6 μg/ml, at 100 mV) to begin to detect single ion channels. In contrast, bilayers containing wild-type sphingolipids required almost two times lower concentration of SRE in the bathing solution. These results suggest that ion-channel formation is energetically more favorable in bilayers containing C4-hydroxylated sphingolipids. It is also possible that the Δ*syr2* non-C4 hydroxylated sphingolipids change the partition coefficient of SRE between the aqueous and lipid phases thereby decreasing the concentration of SRE in the membrane and, consequently, decreasing the likelihood of channel formation.
3. Other conditions being equal, an increase in the concentration of Δ*syr2* sphingolipids in the bilayers results in a decrease in $(dG/dt)_{t=0}$ (Fig. 4). This result indicates that the ability of SRE to form ion channels in the bilayer decreases as the concentration of non-C4 hydroxylated sphingolipids in the bilayer increases. In addition, since no maximum is observed in this curve (Fig. 4) and since sphingolipids from Δ*syr2* inhibit channel formation, it is unlikely that sphingolipids are serving as receptors for SRE. If sphingolipids were serving as receptors for SRE we would likely observe a bell-shaped, concentration-dependent relationship as, for example, has been shown for the glutamate receptor (Lombardi *et al.*, 2001).
4. The effect of field sign (Fig. 2), small channel conductance (Fig. 5), and the effective gating charge were the same for membranes of either wild-type or Δ*syr2* sphingolipid composition. This implies that sphingolipid C4 hydroxylation does not affect the possible structural role of sphingolipids in SRE pore formation nor does it influence the SRE channel gating charge.
5. The unitary conductance of the large SRE channels at positive voltages was 1.5 times greater in bilayers containing wild-type sphingolipid then in Δ*syr2* sphingolipid containing bilayers and remained undistinguishable for both membranes at negative voltages (Fig. 5, curves 2 and 3). Given the possibility that large SRE channels are several synchronously opening small channels, this observation implies that fewer small channels are synchronously opened within a large channel-cluster at positive voltages. In addition the relative number of large SRE channels, *S*, was almost twice as large in wild-type then in Δ*syr2* sphingolipid containing membranes. This suggests that the energetic of SRE cluster opening depends on membrane sphingolipid composition: the presence of non-C4-hydroxylated sphingolipid increases the energy barrier of cluster opening. Moreover, the large channel contribution to the total transmembrane current was 1.6 times larger in the case of wild-type sphingolipid containing membranes. In other words synchronous opening of the small SRE channels more probable when wild-type sphingolipid is present in the membrane. Although these changes in the properties of large SRE channels contribute to the impeded SRE-induced conductance of Δ*syr2* sphingolipid-containing membranes, they are rather modest in and not sufficient to explain the large differences seen in macroscopic current caused by SRE in the two membranes under study (Fig. 2). Therefore, the decrease in SRE channel-forming activity in the presence of Δ*syr2*

sphingolipids in the membrane is mostly due to there being a smaller total number of SRE channels in one membrane as opposed to the other.

Analogous observations have been reported on the effects of sterols on SRE activity in lipid bilayers and red blood cell membranes (Feigin *et al*., 1997; Blasko *et al*., 1998). Cholesterol, but not ergosterol or stigmasterol, impeded SRE channel formation in DOPS/DOPE bilayers, though none of the sterols altered the single channel properties (Feigin *et al*., 1997). Related findings were reported by Blasko *et al*. (Blasko *et al*., 1998) who measured the SRE-induced ^{86}Rb permeability of red blood cell membranes. In these studies, depletion of membrane cholesterol by 50%, or substitution of cholesterol by ergosterol, increased SRE-induced permeability, suggesting that cholesterol inhibited SRE channel formation. These results suggested that the effects of sterols on SRE channel-forming activity could be explained in terms of differences in the partition coefficient of SRE between the aqueous and lipid phases, i.e. the number of SRE channels.

The exact molecular mechanism by which sphingolipids affect SRE channel-forming activity is not known. However, data presented in the study of Lofgren and Pascher (1977) allow us to speculate about interactions between SRE and lipid molecules. This study compared C4 hydroxylated ceramide and with C4 non-hydroxylated ceramide by constructing pressure-area isotherms for Langmuir films. The data showed that C4 hydroxylation promotes condensation of lipid lateral packing in monolayers. This is likely to due to increased hydrogen bonding of the long chain base portions which form the lipid head groups. The relevance of this observation to the current SRE channel formation in the two membrane types is the possibility that increased hydrogen bonding between SRE (the peptide portion and the β hydroxyl of the fatty acid) with the C4 hydroxyl groups increases the partition coefficient of SRE between the lipid and aqueous phases, resulting in the promotion of channel formation.

Taken together, our data suggest that the inhibiting effect of non-C4 hydroxylated sphingolipids is mainly due to its ability to diminish the total number of SRE channels in the membrane, or to decrease the partition coefficient of SRE between the lipid and aqueous phases when compared with the C4 hydroxylated sphingolipids. In addition the presence of non-C4 hydroxylated sphingolipids in the membrane increases the energy barrier for synchronous opening of small channels in the clusters without affecting the properties of elementary (small) SRE channels. These effects provide an explanation for the differential SRE sensitivity of yeast strains that vary only in the degree of sphingolipid C4 hydroxylation.

ACKNOWLEDGMENTS

This study was supported in part by the Russian Foundation for Basic Research (Grant # 03-04-49391); by the Program of Research Schools (# 2178.2003.4); by the Program "Molecular and Cell Biology" of the Russian Academy of Siences; by a grant from the Department of Veterans Affairs, USA (to J.G.B); by Grant 2T32 DC00014 to the Monell Center; and by grants from the Utah Agricultural Experiment Station (Project 607) and Eli Lilly and Co. (to J.Y.T.).

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