J. Membrane Biol. 150, 41–47 (1996)

The Journal of
Membrane
Biology

Properties of Voltage-gated Ion Channels Formed by Syringomycin E in Planar Lipid Bilayers

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Received: 3 April 1995/Revised: 24 August 1995

Abstract. Using the planar lipid bilayer technique we demonstrate that the lipodepsipeptide antibiotic, syringomycin E, forms voltage-sensitive ion channels of weak anion selectivity. The formation of channels in bilayers made from dioleoylglycerophosphatidylserine doped with syringomycin E at one side (1–40 μg/ml) was greatly affected by *cis*-positive voltage. A change of voltage from a positive to a negative value resulted in (i) an abrupt increase in the single channel conductance (the rate of increase was voltage dependent) simultaneous with (ii) a closing of these channels and an exponential decrease in macroscopic conductance over time. The strong voltage dependence of multichannel steady state conductance, the single channel conductance, the rate of opening of channels at positive voltages and closing them at negative voltages, as well as the observed abrupt increase of single channel conductance after voltage sign reversal suggest that the change of the transmembrane field induces a significant rearrangement of syringomycin E channels, including a change in the spacing of charged groups that function as voltage sensors. The conductance induced by syringomycin E increased with the sixth power of syringomycin E concentration suggesting that at least six monomers are required for channel formation.

Key words: Syringomycin E — Ion channels — Voltage gating — Lipid bilayers

Introduction

The syringomycins are cyclic lipodepsipeptides produced by certain strains of the phytopathogenic bacterium *Pseudomonas syringae*pv. *syringae,* (Sinden, DeVay & Backman, 1971; Gross, DeVay & Stadman, 1977). The structure of the most abundant form, syringomycin E, was recently determined (Segre et al., 1988; Fukuchi et al., 1992) (Fig. 1). The syringomycins are virulence factors that have been implicated in a variety of bacterial plant diseases including necroses of stone fruits and holcus spot disease of maize (Gross & DeVay, 1977). They also inhibit the growth of yeast and fungi, an activity that may enhance the survival and competitiveness of *P. syringae* pv. *syringae* on host plant surfaces (Zhang & Takemoto, 1986; Takemoto, 1992).

Plasma membranes are the primary site of action of syringomycin E, but the mechanism of action at this site has not been elucidated (Takemoto, 1992). Among the known effects on yeast and plant plasma membranes are increased cation fluxes, altered H^+-ATPase activities and membrane protein phosphorylation (Zhang and Takemoto, 1986; Bidwai et al., 1987; Takemoto, 1992). In yeast, a normal level of sterols is required for growth inhibition by syringomycin E suggesting an interaction with membrane lipids (Takemoto et al., 1993). Consistent with a membrane interaction mechanism is the report that syringotoxin, a related cyclic lipodepsipeptide, forms anion selective, voltage-sensitive channels in planar lipid bilayers (Pokorny & Ziegler, 1984; Ziegler, Pavlovkin & Pokorny, 1984).

In this paper, using the planar lipid bilayer technique with syringomycin E, we report the formation of anion selective ion channels by syringomycin E and unique voltage sensitivity properties of these channels.

Materials and Methods

Synthetic 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) was obtained from Avanti Polar Lipids, Pelham, AL. All electrolytes were

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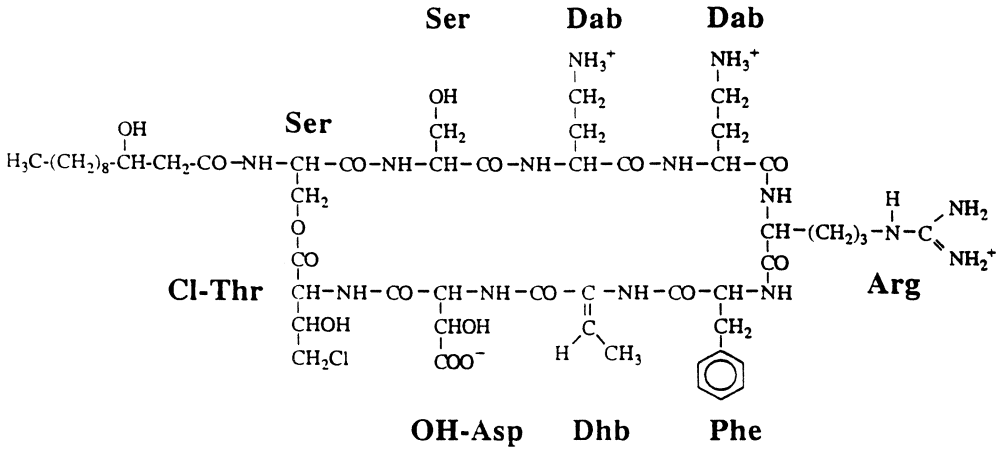

Fig. 1. Syringomycin E (zwitterionic form). Abbreviations: Arg, arginine; OH-Asp, 3-hydroxyaspartic acid; Dab, 2,4-diaminobutyric acid; Dhb, dehydro-2-aminobutyric acid; Cl-Thr, 4-chlorothreonine; Ser, serine; Phe, phenylalanine.

reagent grade. All 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 (Bidwai & Takemoto, 1987).

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 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 membrane preparation and single channel data analysis may be found elsewhere (Teeter et al., 1990; Bezrukov & Vodyanoy, 1993).

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

Results

The Macroscopic Conductance of Bilayers Modified with Syringomycin E

The addition of syringomycin E to the bathing solution at one side of the bilayer induced an increase in bilayer conductance in a voltage-dependent manner. The temporal course of the current across the bilayer in response to, first, a positive voltage step, and then, a negative one (from +120 to −120 mV, from +135 to −135 mV, from +160 to −160 mV, from +170 to −170 mV, from +180 to −180 mV, from +200 to −200 mV) in the bilayer modified with one sided addition of syringomycin E, is depicted in Fig. 2.

The rate of the increase in current that was induced by the application of the positive voltage grew exponentially with the applied voltage. The dependence of the rate of current increase (*dI/dt*), calculated from the data shown in Fig. 2, on the applied positive voltage is shown in Fig. 3. The parameter, *dI/dt*, changes *e*-fold for every 13 mV.

An abrupt change in transmembrane voltage from a positive to a negative value of the same magnitude led to two outcomes: first, an immediate increase in the absolute value of transmembrane current (which was slightly voltage dependent — the relative increase of the current grew from 1.6-fold on going from +120 to −120 mV to 2.0-fold on going from +200 to −200 mV) (the actual values of transmembrane current before and after the reversal of voltage sign are shown in Fig. 2); and second, an inactivation of the membrane conductance. The increasingly prominent ''tail'' of the inactivating portion of the transmembrane current (insert in Fig. 2, *d*) demonstrates that this inactivation of current results from the successive closing of single channels (*see* below for a more detailed description of single-channel activity).

At negative voltages the current decreased as a double exponential; the higher the voltage, the more rapid the decrease of membrane current. The decreasing portion of the current resulting from the negative voltage step is fit by the double-exponential equation

$$I = A_1\exp(-k_1 t) + A_2\exp(-k_2 t).$$

An example is presented as an insert in Fig. 2 *f*, where $k_1 = 0.009\ \text{msec}^{-1}$; $k_2 = 0.001\ \text{msec}^{-1}$; $A_1 = -3270$ pA and $A_2 = -1400$ pA.

The dependence of rate constants k_1 and k_2 (calculated from the kinetics of current decreases shown in Fig. 2) on the applied voltage are shown in Fig. 4. Both parameters were found to be voltage-dependent, changing *e*-fold for every 24 mV for k_1, and every 27 mV for k_2.

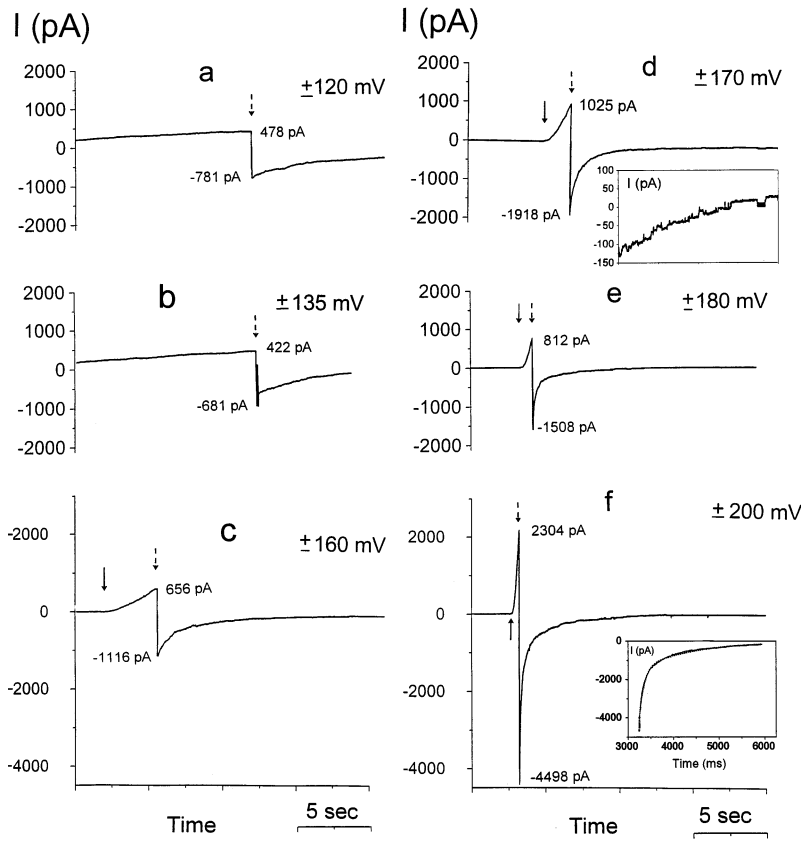

Fig. 2. Time course of the membrane current in field-reversal experiments in the presence of syringomycin E at different voltages. The application of positive voltage (marked by the solid arrows, *c–f*) was followed by a step-like change to negative voltage of the same amplitude (marked by the dotted arrows). The time of application of voltages +120 and +135 mV (*a* and *b*) is not shown due to the slow increase of current. The concentration of syringomycin E (*cis* side only) in the bathing solution (100 mM NaCl, 5 mM MOPS, pH 6.0) was 10 μg/ml. Syringomycin E was added to bathing solution 4 min before the records were taken. The extreme amplitudes of current (pA) before and after the reversal of the electric field are indicated in each panel. Panels *a* through *f* show the effect of increasing voltage application on the magnitude and character of the current response by bilayers incorporating syringomycin E. The insert in panel *d* shows a particular portion of the current kinetics with an expanded scale in the current dimension (the time scale was not changed). This expanded current scale makes visible the single channel fluctuations. The insert in panel *f* shows a portion of the descending current curve fitted by the sum of a two exponential function (*see* text for details). The dotted line is the theoretical curve.

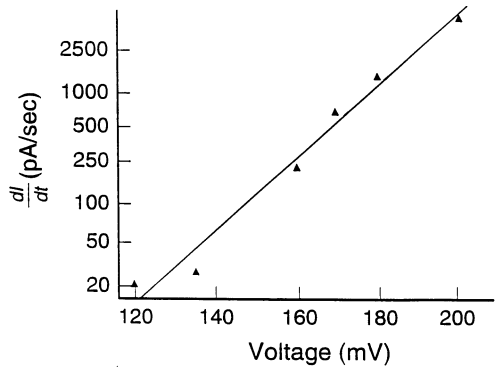

Fig. 3. The dependence of the rate of current increase (*dI/dt*), calculated from data presented in Fig. 2, on the applied positive voltage. The parameter, *dI/dt*, changes *e*-fold for every 13 mV change in voltage.

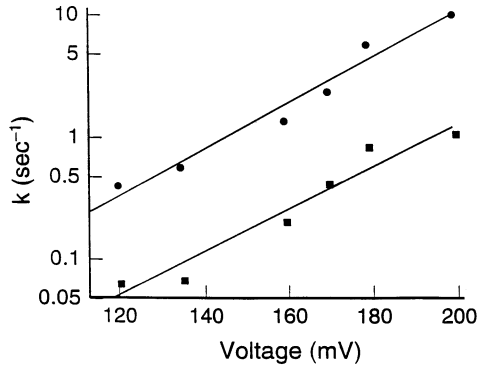

Fig. 4. The dependence of the rate constants k_1 (-o-) and k_2 (-n-) of current decrease, calculated from data presented in Fig. 2, on the applied negative voltage. Both parameters were found to be voltage-dependent, changing *e*-fold for every 24 mV (for k_1), and *e*-fold for every 27 mV (for k_2) change in voltage.

The dependence of steady-state conductance induced by syringomycin E on the applied positive voltage was studied in the narrow range of voltages because we could not obtain steady-state conductance at voltages higher than +50 mV due to an ''infinite'' growth of conductance with time. An example of changes of the steady-state current across the bilayer modified with syringomycin E in response to the stepwise changes (5 mV in the each step) of the amplitude of transmembrane voltage in the voltage range 25–50 mV (positive at *cis* side) is presented at Fig. 5. The plot of the logarithm of membrane steady-state conductance *vs.* applied voltage is presented as an insert to Fig. 5. The steady-state conductance grows exponentially with the applied voltage and changes *e*-fold for every 10.6 ± 0.3 mV. The average value of the voltage change necessary to increase

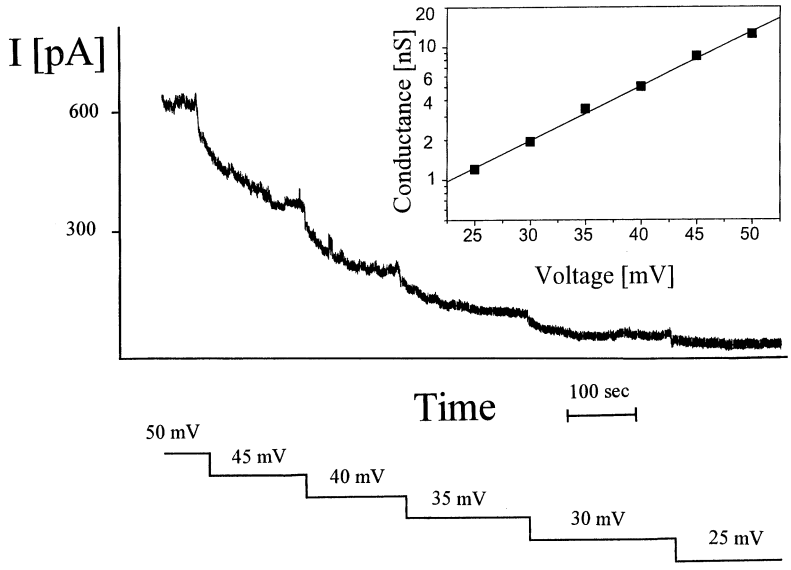

Fig. 5. An example of changes of the steady-state current across a DOPS bilayer modified with syringomycin E (10 μg/ml, *cis* side only) in response to the stepwise changes (5 mV in the each step) of the amplitude of transmembrane voltage in the voltage range 25–50 mV (positive at *cis* side). The stairlike line marks the times of voltage changes. The plot of logarithm of membrane steady-state conductance *vs.* applied voltage approximate a straight line (insert). The slope of the line is related to a change of *e*-fold in conductance every 10.6 ± 0.3 mV.

membrane steady-state conductance *e*-fold was 11.8 ± 3.4 mV (across 5 experiments). This behavior corresponds to an effective gating charge of 2.0 (Latorre & Alvares, 1981; Tosteson & Tosteson, 1984; Hille, 1992).

The dependence of the membrane steady-state conductance on the concentration of syringomycin E in the bathing solution was measured by comparing steady-state conductances at the positive (*cis* side) voltage of +25 mV for 3–4 concentrations of syringomycin E for each bilayer (6 bilayers total). An example of a typical concentration/effect curve is shown in Fig. 6. The conductance increased with the sixth power of syringomycin E concentration. Though the absolute response of certain concentrations of syringomycin varied, the slopes of the curves (logarithm of steady-state conductance versus logarithm of concentration of syringomycin) in all cases were in the range of 5–7 (6.2 ± 0.5).

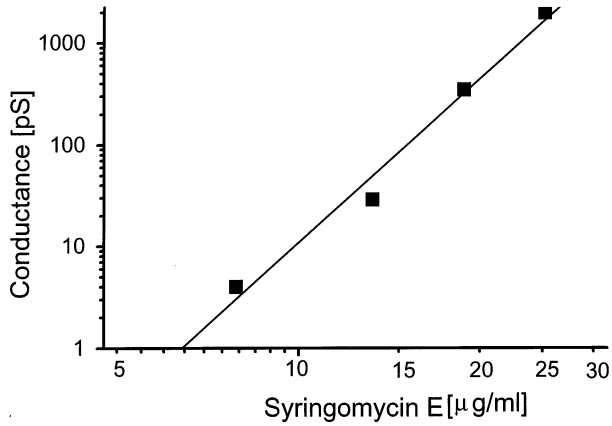

Fig. 6. An example of the dependence of the membrane steady-state conductance on the concentration of syringomycin E in the bathing solution (*cis* side only). A steady-state conductance was measured at a positive (*cis* side) voltage of 25 mV for each concentration.

The Channel-forming Activity of Syringomycin E

After addition of low concentrations of syringomycin E (1.0–5.0 μg/ml) to one side of the bilayer, single-channel activity was observed after a step of positive voltage (150–200 mV). After several channels appeared, the voltage was changed to lower levels, allowing calculation of the current-voltage curves for single channels. Decreases in the level of positive transmembrane voltage resulted in decreases in the number of channels over time without a change in single channel conductance (about 7 pS in 100 mM NaCl).

The records of transmembrane current for bilayers containing a few syringomycin E channels before and after reversal of applied electric field are shown in Fig. 7. The reversal of voltage from positive to negative resulted in (i) a steplike doubling of the macroscopic conductance of the bilayer; (ii) the appearance of channels with high conductance — 2–5 times higher than the conductances of channels at positive voltages (the amplitude of increase was voltage dependent); and (iii) the closing of these channels and an exponential decrease of the number of open channels. The decrease in the number of open channels began immediately after field reversal. In addition to the large channels with high conductance that appeared after the reversal of the field from positive to negative, ''small'' channels, with conductances similar to those of channels observed at positive voltages, were also observed. Both types of channels are clearly visible at negative potential in the ±200 mV record of Fig. 7.

The conductance of single channels was voltage-independent in the case of positive voltages up to +200

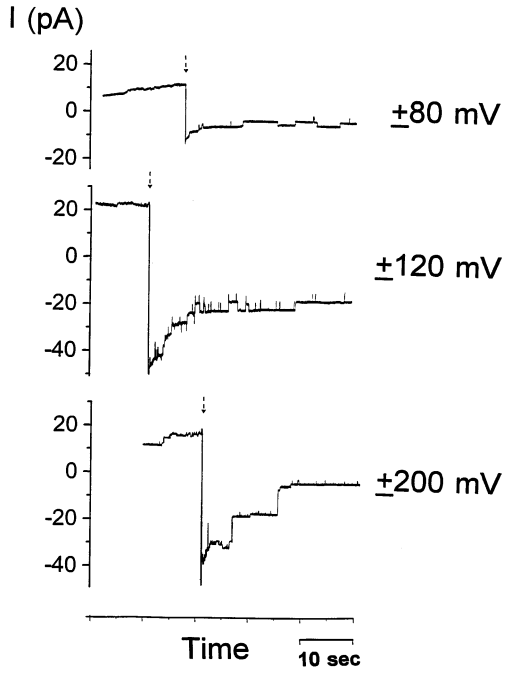

Fig. 7. The records of transmembrane current of bilayers containing a few syringomycin E channels before and after the reversal of applied electric field. The concentration of syringomycin E (*cis* side only) in the bathing solution (100 mM NaCl, 5 mM MOPS, pH 6.0) with 5 μg/ml. Channel openings are represented by upward deflections at positive voltages, and by downward deflections at negative voltages. Data were filtered at 30 Hz, digitized at 5 kHz.

mV (Fig. 8). For negative voltages, the conductance of large single channels increased with voltage. This increase in single channel conductance was consistent with an increase in the macroscopic conductance of bilayers modified with syringomycin E after the reversal of the applied voltage from positive to negative. It is of interest that this increase of single channel conductance was voltage dependent: from 2-fold on going from +80 to −80 mV to 5-fold on going from +200 to −200 mV (Fig. 5). On the other hand, the increase of macroscopic conductance of bilayers was only slightly voltage dependent: from 1.6-fold on going from +120 to −120 mV to 2-fold on going from +200 to −200 mV (Fig. 2). This discrepancy suggests that at high negative voltages the absolute rate of inactivation is sufficiently fast either to prevent the conductance from reaching the maximum value possible in the absence of inactivation, or to prevent the recording of a maximum value with the aperture that we used.

Cation-Anion Selectivity of Syringomycin E Channels

The cation-anion selectivity of syringomycin E channels in DOPS was determined for NaCl. A potential of zero

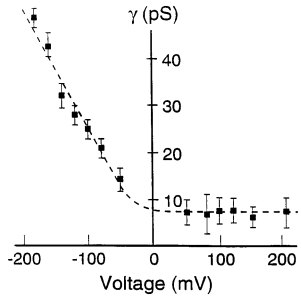

Fig. 8. Voltage dependence of single channel conductances obtained from the records of single channel activity induced by syringomycin E (*cis* side only, 5 μg/ml) in the bathing solution (100 mM NaCl, 5 mM MOPS, pH 6.0).

current (reversal potential) was measured after formation of a 10-fold transmembrane concentration gradient of electrolyte (1 M NaCl at *cis* side and 0.1 M NaCl at *trans* side) across the bilayer modified with sufficient syringomycin E (*cis* side only) to induce a bilayer conductance of 50–1000 pS. The average reversal potential (7 bilayers) was +26 ± 3 mV. This value corresponds to weak anion selectivity, with a cation transfer number of 0.27 and an anion transfer of 0.73. The most probable explanation for such anion-selectivity would be a positive charge (most likely belonging to the protonized amino groups) fixed at the entry of the channel. The sign of selectivity is consistent with the occurrence of three positive charges (two residues of diaminobutyric acid and one residue of arginine) and one negative charge (the residue of 3-hydroxy-aspartic acid) in the peptide portion of the syringomycin E molecule (*see* Fig. 1).

Discussion

Voltage dependency of the macroscopic conductance, the single channel conductance, and the kinetics are well-known phenomena for many channel-formers (Latorre & Alvares, 1981). The voltage-dependent anion channels from the outer membrane of mitochondria (VDAC), reconstituted into lipid bilayers, and alamethicin channels are two well-characterized examples. At the multichannel level, the steady-state VDAC-induced conductivity was decreased by nearly half upon increasing transmembrane voltage from 10 to 50 mV, with a relaxation time of about 10 sec (Colombini et al., 1987). Much higher voltage sensitivity was displayed by alamethicin, which responded to every 10 mV increment with an approximately 10-fold change in its multichannel conductance (Vodyanoy, Hall & Balasubramanian, 1983).

We studied the multichannel voltage sensitivity of the syringomycin-induced conductance using one sided (asymmetric) addition of the antibiotic. Detectable

membrane current developed and increased with time after the application of a positive transmembrane voltage step at the antibiotic (*cis*) side. The reversal of field sign induced a steplike doubling of the absolute value of the membrane current, which was followed by an exponential decrease of current (Fig. 2).

The steplike character of the increase in membrane conductance, after the reversal of transmembrane potential, suggests that this increase derives either from steplike increases of the conductances of single channels, from an increase in the number of channels, or from a synchronization of opening of many channels, without an increase in single channel conductance. The experiments using bilayers containing only a few single channels demonstrated that a reversal of transmembrane potential from positive to negative induced an increase in conductance of single channels in the open state, with a simultaneous decrease of the number of open channels (Fig. 7).

The response of channels induced by syringomycin E to reversal of transmembrane voltage differs from those of voltage-gated channels induced by other well-studied antibiotics and toxins. According to Menestrina et al. (1985) the response to reversal of voltage applied across membranes doped with alamethicin is an abrupt disappearance of all current, followed by an exponential increase in current to a new steady state. An inactivation of membrane conductance after reversal of polarity from positive to negative similar to that observed with syringomycin E was also observed with the polyene-like antibiotic, monazomycin (Heyer, Muller & Finkelstein, 1976) and the colicins (Wilmsen, Pugsley & Pattus, 1990; Qiu et al., 1994). However, in these latter cases neither the macroscopic conductance of the bilayers nor the conductance of single channels increased, as is observed with syringomycin E-induced channels. The weak anion-selectivity of syringomycin E channels suggests the occurrence of positive charges (most likely belonging to protonized amino groups) fixed at the entry of the channel.

The rate of increase of conductance with positive voltage steps and the rate of decrease of conductance with negative voltage steps as well as the steady-state conductance of bilayers modified with syringomycin E were exponentially dependent upon the magnitude of the applied voltage (Figs. 3–5). The exponential growth of multichannel steady-state conductance with the applied positive voltage (*e*-fold for every 12 mV) corresponds to an effective gating charge of 2.0 (Latorre & Alvares, 1981; Tosteson & Tosteson, 1984; Hille, 1992). This observation suggests that several elementary charges are involved in opening channels at positive potentials. (It is impossible to calculate the exact number of elementary charges, because the effective charge is a product of the charge translocated and the unknown fraction of the membrane thickness that the charge travels when the channel changes from one state to another (Latorre & Alvares, 1981).)

The voltage dependence of (i), the multichannel steady-state conductance (Fig. 5) (ii), the single channel conductance (Fig. 8) (iii), the rate of opening of channels at positive voltages (Fig. 3) and closing them at negative voltages (Fig. 4), as well as (iv), the observed abrupt increase of single channel conductance after voltage sign reversal (Fig. 7) suggest that the change of the transmembrane field induces a significant rearrangement of syringomycin E channels, including a change in the spacing of the charged groups that function as voltage sensors.

We observed that the conductance induced by syringomycin E increased with the sixth power of syringomycin E concentration. This fact suggests that at least six monomers are needed to form the channel (Finkelstein & Holz, 1973; Latorre & Alvares, 1981). The range of active concentrations of syringomycin E (1–40 μg/ml) is much lower than the critical micelle concentration of this compound (1.25 mg/ml) (*personal communication*, D.C. Gross). Taken together these facts suggest that syringomycin E inserts into the lipid bilayer from the bathing solution as separate molecules which then aggregate into pore complexes.

To explain the unusual voltage-dependent behavior of syringomycin E-induced channels, we suggest the following preliminary model. We assume that the channel is formed by a complex of several (at least, six) molecules of syringomycin. It is also assumed that the lipophilic end of each antibiotic molecule rests in the core of the bilayer, while the more hydrophilic peptide portion is disposed close to the hydrophilic surface of the membrane, on that side of the membrane which received the antibiotic. A positive voltage maintains the antibiotic inside the membrane and, simultaneously, opens a low conductance state of the channel. Reversal of the transmembrane field switches the channels to a new state with higher conductance, and simultaneously induces dismantling of the channel assemblies by pulling the positively charged antibiotic molecules out of the membrane.

In yeast cells, membrane sterols are required for inhibition by syringomycin E (Takemoto et al., 1993). How sterols might influence channel formation induced by syringomycin E remains a question for future study.

The authors are very grateful to Drs. Sergey M. Bezrukov and Igor Vodyanoy for fruitful discussions and advice in the preparation of this manuscript. This study was supported in part by National Institutes of Health grants DC-00356, DC-01838, NSF grant IBN-9003398, and a grant from the Department of Veterans Affairs.

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