Syringomycin E Channel: A Lipidic Pore Stabilized by Lipopeptide?

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ABSTRACT Highly reproducible ion channels of the lipopeptide antibiotic syringomycin E demonstrate unprecedented involvement of the host bilayer lipids. We find that in addition to a pronounced influence of lipid species on the open-channel ionic conductance, the membrane lipids play a crucial role in channel gating. The effective gating charge, which characterizes sensitivity of the conformational equilibrium of the syringomycin E channels to the transmembrane voltage, is modified by the lipid charge and lipid dipolar moment. We show that the type of host lipid determines not only the absolute value but also the sign of the gating charge. With negatively charged bilayers, the gating charge sign inverts with increased salt concentration or decreased pH. We also demonstrate that the replacement of lamellar lipid by nonlamellar with the negative spontaneous curvature inhibits channel formation. These observations suggest that the asymmetric channel directly incorporates lipids. The charges and dipoles resulting from the structural inclusion of lipids are important determinants of the overall energetics that underlies channel gating. We conclude that the syringomycin E channel may serve as a biophysical model to link studies of ion channels with those of lipidic pores in membrane fusion.

INTRODUCTION

Transient lipidic or protein-lipidic pores are involved in many cellular and subcellular processes that include exocytosis, viral fusion, and trafficking (Zimmerberg and Chernomordik, 1999). Recent results on protein-mediated membrane fusion of vesicles strongly suggest that the terminal phase of membrane fusion is realized through special proteolipid channels that expand and incorporate more lipids in their structure in a Ca^{+2}-dependent manner (Peters et al., 2001; Almers, 2001). Among new possible roles for lipidic pores is participation of short- and long-chain ceramides in apoptotic regulation (Siskind and Colombini, 2000).

The important problem of lipidic pore energetics, first addressed more than 20 years ago (Abidor et al., 1979), is still vigorously discussed (May, 2000; Zimmerberg, 2001). Many physical parameters, such as membrane surface tension, hydration, bending modulus, and spontaneous lipid curvature contribute to the pore formation energy. The pore energy balance defines the probability of pore formation and, therefore, determines its role and the mechanism of its regulation in a specific cellular event.

Here we analyze well-defined and highly reproducible channels formed in planar lipid bilayers by a natural lipopeptide, syringomycin E (SRE). In their transport properties, the SRE channels exhibit a number of interesting features. The most unique one is a significant involvement of lipids of host membranes. The membrane composition not only influences open channel conductance to an extent that exceeds most known examples; as we presently show here, the membrane lipids also directly participate in the channel gating.

SRE is a phytotoxin of the cyclic lipodepsinonapeptide class, which is produced by the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae.* The SRE molecule is composed of the polar peptide head and the hydrophobic 3-hydroxyfatty dodecanoic acid tail. The polar head is a macrocyclic lactone ring containing nine amino acid residues, three of which have positive charges, whereas one has negative charge (Segre et al., 1989; Fukuchi et al., 1992; Bender et al., 1999). The primary biological target of SRE action is the plasma membrane. It was shown that SRE promotes passive fluxes of mono- and divalent ions across cell plasma membranes (Reidl and Takemoto, 1987; Zhang and Takemoto, 1987; Reidl et al., 1989) and forms channels in bilayer lipid membranes (Ziegler et al., 1984; Pokorny and Ziegler, 1984; Ziegler et al., 1986; Hutchison et al., 1995). The amphipathic structure of SRE facilitates its insertion into the membrane lipid bilayer.

Abundant information on the SRE channels has been accumulated for the last few years. The SRE channels are preferentially permeable to anions (Feigin et al., 1996; Schagina et al., 1998; Kaulin et al., 1998) and their lumen radius is ~1 nm (Hutchison et al., 1995; Kaulin et al., 1998; Dalla Serra et al., 1999). At least six SRE molecules are required for channel formation (Feigin et al., 1996; Dalla Serra et al., 1999; Malev et al., 2000). Two types of channels, "small" and "large," differing 6 to 7 times in their conductance, were observed (Schagina et al., 1998; Kaulin et al., 1998). At one-sided antibiotic addition, both the kinetics of the channel opening (or closing) and the channel conductance are strongly voltage dependent and pronouncedly asymmetric (Feigin et al., 1996; Schagina et al., 1998; Malev et al., 2000).

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To clarify the reasons for the asymmetrical lipid-dependent voltage sensitivity of the SRE-induced channels, we conducted both single-channel experiments and multi-channel voltage-jump relaxation measurements. Channel behavior was observed as a function of the transmembrane potential, salt concentration of the bathing solution, and lipid composition of the host membranes. We studied two groups of effects. The first group is related to the influence of lipid molecules on conductance of the single SRE channel. The second group addresses their influence on channel gating.

At neutral pH and moderate salt concentrations (e.g., 0.1 M), a marked difference in the SRE channel conductance was observed using neutral and charged membrane-forming lipids. Changing the contribution of the lipid surface charge by using either solutions of increased salt concentrations or neutralizing the charge with the increased solution acidity, we conclude that the observed difference in the channel conductance results from the charge effect on local ionic concentrations and not from possible changes in the size of the channel lumen. Even more importantly, the transition from charged to neutral lipids, as well as the titration of the membrane charge and its screening, is accompanied with the inversion in the sign of the applied potentials that open the SRE channels. Similar modification of the channel gating could be achieved in experiments with phloretin, which is known to compensate lipid dipole moment (Melnik et al., 1977; Cseh and Benz, 1999). In the case of neutral bilayers addition of phloretin is able to reverse the sign of transmembrane potentials opening (or closing) the SRE channels.

Thus, our findings suggest a significant structural involvement of lipid molecules in the channel structure. Tentatively, the SRE channel can be seen as a lipidic pore stabilized by a ring of several lipopeptide molecules.

MATERIALS AND METHODS

The lipids used in this study, the synthetic 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC), were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). All electrolytes were of reagent grade (Sigma, St. Louis, MO). Water was deionized and double distilled. Salt solutions for bilayer experiments were in the range of 0.01 to 2.5 M NaCl. All solutions were buffered by 5 mM MOPS in the range of pH from 2.0 to 6.0. Syringomycin E was purified as described previously (Bidwai et al., 1987). Phloretin was purchased from Sigma.

The solvent-free membranes were prepared as described by Montall and Muller (1972). The membrane-forming solutions were DPhPC, DOPE, DOPC, and an equimolar mixture of DOPS and DOPE in hexane. Two symmetrical halves of a Teflon chamber with solution volumes of 1 to 1.5 cm^3 were separated by a 15-μm-thick Teflon partition containing a round aperture of ~100-μm diameter. Hexadecane in *n*-hexane (1:10, v/v) was used for aperture pretreatment. A pair of Ag-AgCl electrodes was used to maintain the membrane potential and to detect ion currents. The term "positive voltages" means that the *cis*-side compartment (the side of antibiotic addition) is positive with respect to the *trans*-side.

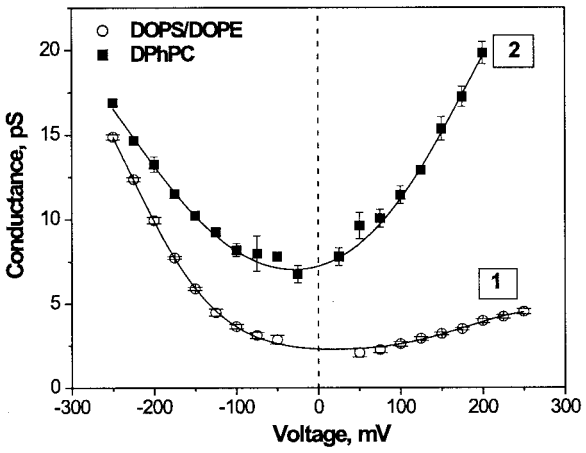

FIGURE 1 Single channel conductances in DOPS/DOPE (curve 1) and DPhPC (curve 2) membranes demonstrate significant lipid involvement and asymmetrical organization of the SRE channels. Higher channel conductance in neutral DPhPC membranes reflects an impact of negative charges of DOPS lipids on ion flow through the SRE channels. Membrane-bathing solutions contained 0.1 M NaCl at pH 6 (5 mM MOPS). The data points were obtained from current histograms; at the least 100 channels were analyzed for each point.

SRE was added to the aqueous phase after the bilayer formation from water stock solutions. The total SRE concentration in the membrane bathing solution did not exceed 0.03 mM. All the experiments were performed at room temperature. The methods used for the membrane preparation and the single channel data analysis are described in detail elsewhere (for example, see Bezrukov and Vodyanoy, 1993).

The mean values of the current, I, through single channels were obtained from current histograms. For each current level, a current amplitude histogram was fitted with the Gaussian distribution using the "Origin" software (Microcal Software, Inc., Northampton, MA). Current-voltage data are presented as integral channel conductance $G = I/V$ as a function of the membrane potential, V.

The channel gating charge was measured in voltage-jump experiments. To determine the number of the SRE channels opened at a given membrane potential, the recorded steady-state current was divided by the corresponding (also voltage-dependent) current through a single channel. This procedure gave us the total number of the open small channels, N_{ch}, which was then used to obtain the effective gating charge, Q.

Although the small channels were mainly examined in this study, the main conclusions are also valid for the large channels. Their conductance differs approximately sixfold independently of the applied potential or bathing solution composition (Schagina et al., 1998; Kaulin et al., 1998), and their gating properties appear to be close (see below).

RESULTS AND DISCUSSION

Single-channel conductance

SRE single-channel conductance was measured as a function of the applied potential, V, in 0.1 M NaCl (pH 6) for the negatively charged (DOPS/DOPE) and neutral (DPhPC) membranes (Fig. 1). The channel conductance strongly depends on the membrane lipid composition: conductance interpolated to zero current conditions ($V \rightarrow 0$) in neutral bilayers is three times higher than that in charged bilayers.

This effect is similar to the lipid charge influence on conductance previously reported for model channels, gramicidin A (Apell et al., 1979; Rostovtseva et al., 1998) and alamethicin (Aguilella and Bezrukov, 2001), but is different in its direction. Oppositely to gramicidin A and alamethicin, the negative charge of the DOPS headgroups decreases the SRE channel conductance. The current-voltage curves of the SRE channel are superlinear in the applied potential and asymmetrical in its sign. In the case of charged lipids (DOPS/DOPE) that gave more stable membranes than neutral lipids, this asymmetry was observed for at least 6 to 7 h. Therefore, the SRE molecules are incorporated in the channel structure asymmetrically and do not penetrate through the membrane easily.

The increased channel conductance in DPhPC membranes compared with that in DOPS/DOPE membranes might be explained by assuming either one or both of the following possibilities: 1) the radius of the channel lumen in DPhPC bilayers exceeds that of the channel in DOPS/DOPE membranes; 2) the negative lipid charge has a direct effect on channel conductance. In the first interpretation, which is purely geometrical, the threefold increase in the channel conductance would correspond to the 1.7-fold increase in the channel radius. Because the SRE channel radius in DOPS/DOPE (0.1 M NaCl, pH 6) was estimated to be ~1 nm (Schagina et al., 1998; Kaulin et al., 1998; Dalla Serra et al., 1999), it might be suggested that the expected increase in the channel radius in the DPhPC membranes up to ~2 nm should decrease the channel selectivity for ions. However, in reality, this is not the case. Our measurements show that the channel anion selectivity in the DPhPC membranes is higher ($t_{Cl^-} = 0.97 \pm 0.02$) than that in DOPS/DOPE membranes ($t_{Cl^-} = 0.77 \pm 0.01$).

Additional evidence against this purely geometrical interpretation was obtained in experiments with varying electrolyte concentration. Fig. 2 shows channel conductance in the limit of $V \rightarrow 0$ as a function of the electrolyte concentration for DOPS/DOPE and DPhPC membranes. Channel conductance in charged bilayers is practically proportional to the electrolyte concentration (curve 1), whereas in neutral bilayers it is approximately proportional to the square root of electrolyte concentration (curve 2). Thus, the screening of the charge on SRE molecules is evident only for uncharged membranes but seems to be absent for the charged ones. The latter may be a manifestation of a compensatory effect where the screening of SRE positive charges and lipid negative charges works in opposite directions.

As electrolyte concentration is increased to 1 M NaCl, channel conductances in charged and neutral lipids converge to the same value. In the limit of $V \rightarrow 0$ it is ~20 pS (Fig. 2). This conductance is approximately two orders of magnitude lower than the conductance calculated from Ohm's law if the channel radius is taken to be equal to 1 nm. The 1-nm estimate for the channel radius was obtained by at least three different methods (Schagina et al., 1998; Kaulin et al., 1998; Dalla Serra et al., 1999; Agner et al., 2000). A similar large discrepancy between the measured and calculated values of the channel conductance was reported for colicin E1 (Raymond et al., 1985; Kayalar and Duzgunes, 1986; Slatin, 1988, Bullock et al., 1992) and, recently, for colicin Ia channels (Krasilnikov et al., 1998). This allows us to suggest that electrostatic interactions between ions and the pore significantly hinder ion movement within these channels through mechanisms that are not presently understood.

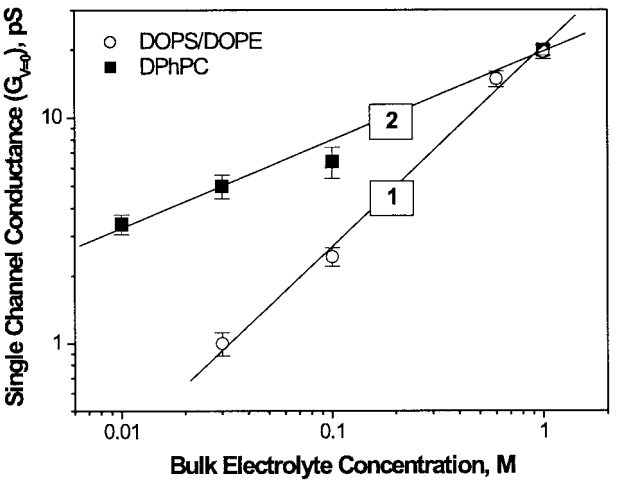

FIGURE 2 Concentration dependence of the single-channel conductance interpolated to zero current conditions ($V \rightarrow 0$) is modified by lipid charge. Channel conductance in DOPS/DOPE membranes scales approximately linearly with NaCl concentration, whereas in DPhPC membranes it is roughly proportional to $[NaCl]^{1/2}$. Note that at the high NaCl concentrations (~1 M) single channel conductances in both neutral and charged lipids converge to the same value. All experiments were carried out at pH 6.

Channel gating

The effect of lipid charge on channel conductance and its dependence on salt concentration suggest direct lipid incorporation into the pore structure. However, there is another, and probably much more important, aspect of the lipid functional involvement. The transitions from charged to neutral (or screened) lipids are accompanied by strong changes in the effective gating charge including inversion in the sign of potentials that open (or close) the channels.

Typical tracks of the transmembrane currents obtained in the field-reversal experiments reveal that the SRE channels have well-defined and highly reproducible unitary conductances (Fig. 3). Positive potentials can either open or close the channels depending on membrane lipid composition, salt concentration, and pH. With DOPS/DOPE membranes in 0.1 M NaCl at pH 6, application of positive potentials opens the channels (Fig. 3 *A*). Appli-

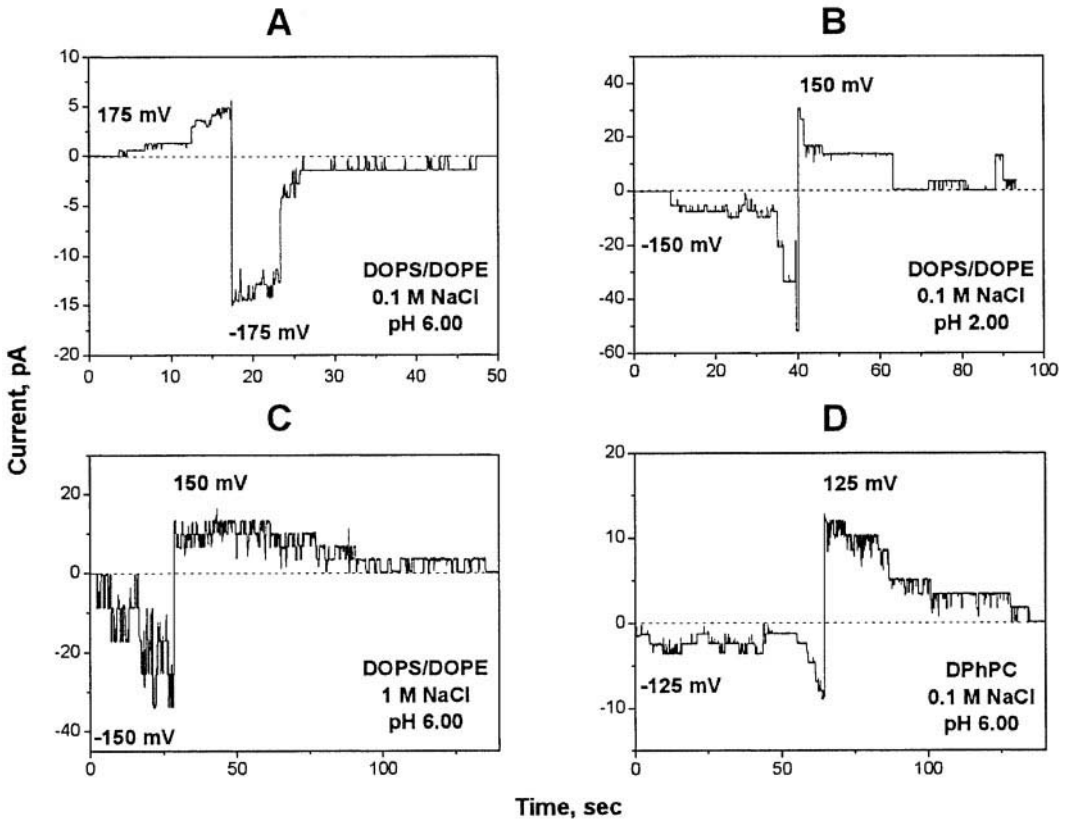

FIGURE 3 Current traces obtained in field-reversal experiments illustrate the effects of membrane lipid composition, bathing solution concentration, and pH on the sign of potentials that open (or close) the SRE channels. For DOPS/DOPE membranes bathed by 0.1 M NaCl at pH 6 (*A*), positive voltages increase the number of open channels. On the contrary, at pH 2 but other conditions being equal (*B*), negative voltages open the channels. The similar sign inversion can be observed at the NaCl concentration increase from 0.1 to 1.0 M (*C*). Removing lipid charge by substituting DOPS/DOPE with DPhPC (*D*) changes the sign of the gating potential as well.

cation of negative potentials, on the contrary, closes the channels. At neutral pH values similar channel behavior was observed with NaCl concentrations ranging from 0.01 to 0.3 M. Decreasing the pH of the bathing solution from 6 to 2 reverses the signs of the opening/closing potentials (Fig. 3 *B*). The sign reversal was also observed at neutral pH as a result of the 10-fold increase in the NaCl concentration (Fig. 3 *C*). In addition, at pH 6 and 0.1 M NaCl the sign reversal takes place when charged DOPS/DOPE bilayers are substituted with neutral DPhPC bilayers (compare Fig. 3, *A* and *D*). This points to a crucial role of the lipid charges in channel gating.

Direct involvement of lipid charge in the voltage-sensitive conformational equilibrium of the channel is further supported by the following observations. First, we did not detect any difference in both the single-channel conductance and gating upon replacing DPhPC with DOPC. Second, in the case of neutral lipids, we did not find the pH-dependent changes in channel gating (data not shown).

Thus, the gating properties of the SRE channels strongly depend on the host lipid charge. By changing lipid species it is not only possible to change the absolute value of the gating charge, but also its sign. The voltage that opens the SRE channels reconstituted in a membrane of a certain lipid composition closes them in a membrane of a different lipid composition. To our knowledge, this finding is unprecedented.

Obviously, the work of channel formation (or opening) includes the Coulomb component. Using the steady-state current dependence on the transmembrane potential, one can determine the dimensionless gating charge Q that characterizes the effect of the electric field on the conformational equilibrium of the SRE channels. The average number of open channels under steady-state conditions, N_{ch}, is related to the work of channel formation, W_{ch}, by Hodgkin and Huxley (1952), Ehrenstein et al. (1970), and Hille (1992):

$$N_{ch} = \frac{N_t}{1 + \exp(W_{ch}/kT)} \quad (1)$$

in which N_t is the total number of channels, and k and T have their usual meanings of the Boltzmann constant and the absolute temperature. Apart from the structural compo-

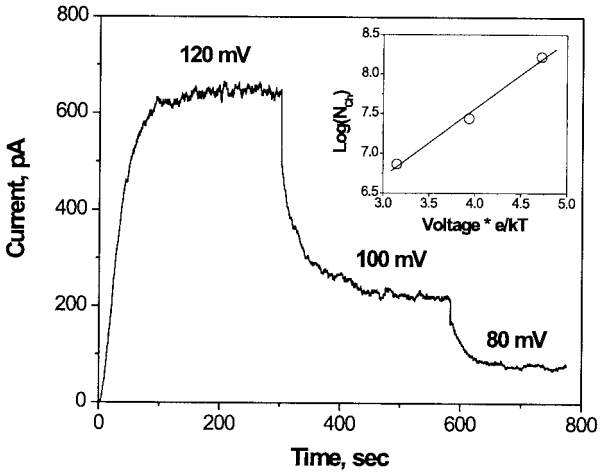

FIGURE 4 Time dependence of the current through the multichannel membranes at the successive changes of the applied voltage from 0 to 120 mV, from 120 to 100 mV, and from 100 to 80 mV. Inset gives a logarithmic plot of the average number of the open SRE channels as a function of the applied voltage. The number of channels was obtained from the steady-state parts of the recordings using data on single-channel conductance (Fig. 1). Membrane-bathing solution was 0.05 M NaCl at pH 6. Lipid bilayer was formed from DOPS/DOPE.

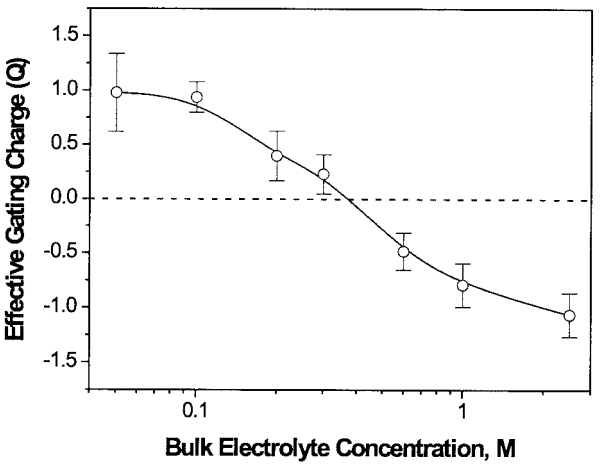

FIGURE 5 The effective gating charge of the SRE channels (Q) strongly depends on NaCl bulk concentration. As the concentration was increased, the gating charge changed from 1.0 to −1.0 with $Q = 0$ at approximately [NaCl] = 0.4 M. Data were obtained for DOPS/DOPE bilayers at pH 6.

nent U_{ch}, the work W_{ch} includes the Coulomb component proportional to the product of the dimensionless gating charge, elementary charge, e, and transmembrane voltage, V. This component results from displacements of charged and/or dipole particles of any origin during channel formation and their interaction with the intramembrane electric field. Linearity in voltage means that the applied field does not change the channel structure (including positions of the charges) per se, it only influences the equilibrium between different structures. An additional component that is proportional to voltage squared may also be included into the channel formation work to account for possible electrostriction. Thus, the work W_{ch} is given by the equation:

$$W_{ch} = U_{ch} - eQV - \alpha V^2. \quad (2)$$

In the case of the SRE channels we do not see the saturation in N_{ch} predicted by Eq. 1. This means that under the conditions of our experiments, the inequality $W_{ch} \gg kT$ is fulfilled, and therefore

$$N_{ch} \propto \exp\left(\frac{eQV + \alpha V^2}{kT}\right) \quad (3)$$

The gating charge Q and "electrostriction parameter" α can be obtained from the dependence of the number of open channels on the transmembrane potential.

Typical results of a voltage-jump experiment and the corresponding dependence of the average number of open channels on applied voltage are shown in Fig. 4. The average number of channels was calculated from steady-state currents after ~100 s relaxation to a new current level using single channel conductances obtained in independent experiments (Fig. 1). The number of channels was found to be exponential in the applied voltage (Fig. 4, inset). Thus, the work of the channel opening is linear in the voltage. We conclude that the electrostriction force responsible for the αV^2 component does not significantly affect the channel opening and, therefore, the gating charge can be found as

$$Q = \frac{d(\ln N_{ch})}{d(eV/kT)}. \quad (4)$$

The gating charge of the SRE channels in DOPS/DOPE membranes is close to 1.0 for NaCl concentrations not exceeding 0.1 M (Fig. 5). However, as the electrolyte concentration is increased, the gating charge first decreases and then changes its sign reaching −1.0 at 2.5 M NaCl. The transition from positive to negative values occurs at ~0.4 M.

In 0.1 M NaCl a similar inversion of the gating charge sign is achieved by increasing the solution acidity. Change of bathing solution pH from 6 to 2 leads to a decrease in Q from ~0.8 to −0.4 with the transition point around pH 3 (Fig. 6). Therefore, both lipid charge titration by protons and screening by the increased salt concentration lead to qualitatively similar effects: an initial decrease and then sign inversion of the channel gating charge. Importantly, with neutral lipids the gating charge is negative already at low salt concentrations (Fig. 3 *D*). These observations reveal the critical participation of lipid molecules in the channel structure. Channel opening (or formation) involves translocation of charged lipid heads along electric field lines probably implying that lipids are essential building components of the SRE channel pore.

A highly simplified cartoon of the hypothetical channel structure that incorporates membrane lipids is given in Fig. 7. The structure is asymmetric with the antibiotic lactone

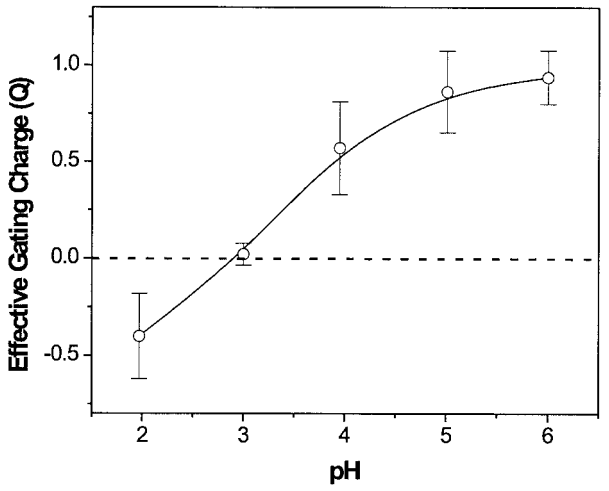

FIGURE 6 The effective gating charge of the SRE channels can be modified by bathing solution pH. Decreasing membrane-bathing solution pH from 6 to 2 we change the gating charge from ~0.8 to −0.4 with Q = 0 at approximately pH 3. Data were obtained for DOPS/DOPE bilayers in 0.1 M NaCl solutions.

ring located closer to the *cis* side (the side of SRE addition) than to the *trans* side. We assume that the SRE channel includes six to seven antibiotic molecules encircling the ion conductive pore. This is based on the fact that the integral conductance of SRE-modified membranes is proportional to the 6 to 7 power of the antibiotic concentration in the bathing solution (Feigin et al., 1996; Dalla Serra et al., 1999; Malev et al., 2000). Within the studied range of SRE concentrations, only monomer particles of the antibiotic are present in aqueous solutions (Dalla Serra et al., 1999).

The channel asymmetry accounts for the observation that with negatively charged bilayers (DOPS/DOPE) and dilute bathing solutions ([NaCl] ≤ 0.3 M), the opening of the SRE channels is favored by positive transmembrane potentials. Pore formation requires a larger number of negatively charged lipid headgroups to move down the electric field than up the field. If the applied potential is such that the *cis* side of the membrane is more positive, then the lipid headgroups lining the *trans*-side opening of the pore are dragged into the pore reducing the work of the channel formation. The lipid headgroups located near the *cis*-side opening would act in the opposite direction. However, due to the channel asymmetry their total number is smaller, so that the energy contribution of headgroups at the *trans*-side opening will dominate.

The sign of potentials favoring channel opening in such systems can be reversed by using neutral lipids (Fig. 3 *D*) or by increasing solution acidity to pH 2 (Fig. 6). Both maneuvers eliminate the charge of the lipid headgroup. The remaining voltage dependence of channel gating can be attributed to the charges on the antibiotic molecule itself (z_{ef} = 2 per molecule) and/or dipolar moments of lipid headgroups.

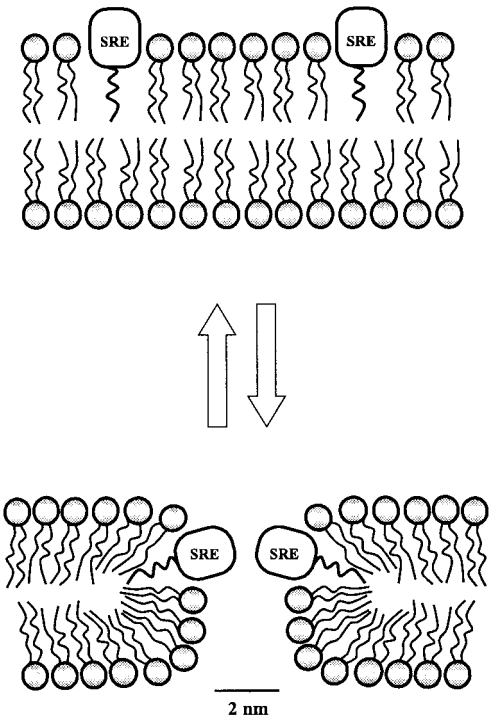

FIGURE 7 A simple model accounting for the unprecedented involvement of the membrane lipids in the channel functional properties. The proposed structure is simply a lipidic pore stabilized by a ring of SRE molecules. Asymmetrical shape reflects asymmetry of the channel found in experiments. Upper part corresponds to the *cis* compartment of the chamber, that is, the side of antibiotic addition.

Dipole contribution to channel gating

To estimate the dipole contribution to channel gating, we use the model in Fig. 7 with the channel length and radius equal to 6 and 1 nm, respectively. We assume that the *trans* side of the channel (3 nm in length) is built of lipid molecules only, whereas the *cis* side contains a ring of six SRE molecules occupying 2 nm of the channel length. Using the area of a lipid molecule in a monolayer (≈0.7 nm^2), we suggest that the channel is composed of three rings of lipids at its *trans* side and one ring of lipids at its *cis* side, each ring consisting of ~10 molecules. Thus, the total number of lipids in the channel structure is estimated to be 40. We also assume that the dipole vector of a lipid molecule is always parallel to the surface that this molecule forms (Seelig, 1978; Frischleder and Peinel, 1982; Gawrisch et al., 1992).

The problem is complicated by uncertainties in lipid headgroup orientation within the pore structure. If we neglect the field inhomogeneity at distances comparable to the size of the lipid headgroup, the contribution from lipid dipoles can be written as a negative sum of scalar products of the time-averaged dipole vectors by the local fields $-\langle \mathbf{d}_n \rangle \mathbf{E}_n$. This is the work that is required to bring dipoles from positions where the electric field is zero to positions where the field acting on dipole n is $\mathbf{E}_n$.

We consider two cases: the limit of highly ordered and the limit of disordered headgroup dipoles. To obtain an upper estimate for the highly ordered case, we assume that because of their interactions with the SRE molecules, the dipoles of the *cis* and *trans* lipids in the pore are antiparallel to each other and are aligned along the transmembrane field. The antiparallel orientation leaves an excess of two lipid rings in the pore structure, which gives ~20 uncompensated lipid dipoles. Taking 20 Debye for each headgroup dipole moment (Seelig, 1978; Frischleder and Peinel, 1982), or $\sim 7 \times 10^{-29}$ C·m, and 2×10^7 V/m for the transmembrane field (corresponding to 100 mV of applied voltage), we obtain 3×10^{-20} J, the work that exceeds 5 kT at room temperature.

For the case of completely disordered headgroups, the dipoles undergo free thermal rotation in the plane parallel to the local surface. Their predominant orientation is induced by the transmembrane field, so that $\langle \mathbf{d}_n \rangle = 0$ in the absence of an applied potential. The Boltzmann factor of the field-induced orientation for the dipoles rotating in a plane parallel to the transmembrane field is $\exp(|\mathbf{d}_n| E_h \cos(\varphi)/kT)$, in which φ is the angle between the dipole and the field. Therefore, the absolute value of the time-averaged dipole is given by

$$|\langle \mathbf{d}_n \rangle| = |\mathbf{d}_n| \frac{\int_0^\pi \cos(\varphi) \exp(|\mathbf{d}_n| E_n \cos(\varphi)/kT) d\varphi}{\int_0^\pi \exp(|\mathbf{d}_n| E_n \cos(\varphi)/kT) d\varphi} \quad (5)$$

For a field of 4×10^7 V/m and a dipole of $|\mathbf{d}_n| = 20$ Debye, Eq. 5 gives a significant average for the dipole projection, $|\langle \mathbf{d}_n \rangle| \cong 7$ Debye. Taking into account the total number of lipid molecules in the channel structure, this leads to an energy estimate of several kT. However, analysis of Eq. 5 shows that for transmembrane potentials in the range from −100 mV to + 100 mV, $|\langle \mathbf{d}_n \rangle|$ is approximately linear in the applied voltage, so that the sum $\langle \mathbf{d}_n \rangle \mathbf{E}_n$ is quadratic in the applied voltage. Thus, the case of disordered dipoles gives a nonlinear dependence of the pore-formation energy on the applied field. As discussed above, this conjecture contradicts our experimental findings (Fig. 4).

A reliable estimate for the lipid dipole component requires more precise structural information. However, it is seen that the energy of headgroup dipole interaction with the transmembrane field is large enough to be measurable in our gating experiments. If so, then dipole-modifying agents should influence channel gating. They do. Experiments with phloretin, which compensates lipid dipole moment by aligning its own dipoles in the opposite direction (Melnik et al., 1977; Cseh and Benz, 1999), clearly demonstrated this influence (Schagina et al., 2001). We found that addition of phloretin to neutral lipid membranes inverts the sign of the SRE channel gating charge (data not shown).

Effects of lipid spontaneous curvature

The data discussed above imply that the host membrane lipids are essential components of the SRE channel structure. The model in Fig. 7 represents the channel as a lipidic pore where antibiotic molecules play the role of a stabilizing structure that defines the pore size. The formation of such a lipidic pore necessarily involves the work of lipid monolayer bending (Chernomordik et al., 1985; May, 2000) up to many hundred kTs (Kuzmin et al., 2001; Fuller and Rand, 2001). In the case of the SRE channels, this high-energy cost is probably partially compensated by the attractive interactions between the antibiotic molecules in the SRE ring. However, even with this compensation, the channel structure energetics have to be sensitive to the mechanical properties of lipid molecules. One of these properties is characterized by the lipid spontaneous curvature reflecting "effective molecular shape" of the lipid (Luzzati and Husson, 1962; Cullis and de Kruijff, 1979, Chernomordik et al., 1985; Gruner, 1985).

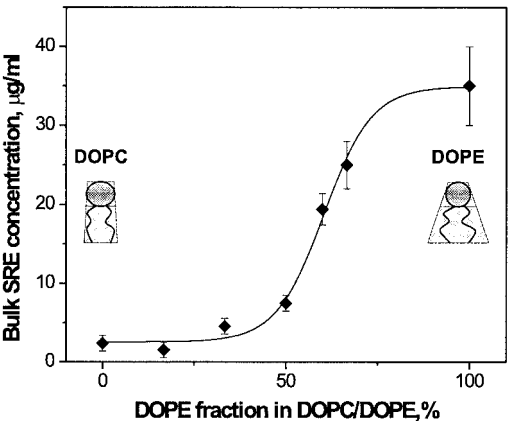

FIGURE 8 The increase of the nonlamellar lipid fraction (DOPE) in DOPC/DOPE membranes inhibits channel formation. Specifically, in pure DOPE membranes a 15-fold higher SRE bulk concentration is required to form single channels as compared with pure DOPC membranes. Insets illustrate difference in "molecular shapes" of nonlamellar DOPE and lamellar DOPC. The membrane-bathing solution was 0.1 M NaCl at pH 6. Single-channel activity was obtained at the applied voltage of −175 mV within 10 min after SRE had been added to the solution in the *cis* compartment.

To study the influence of the lipid shape we used mixtures of two neutral lipids: DOPE and DOPC. In excess water DOPE spontaneously forms nonlamellar inverted hexagonal phases, whereas DOPC normally forms lamellar phases. It is well known that nonlamellar lipids affect the activity of membrane proteins and peptides (Epand, 1998; Bezrukov, 2000). The probabilistic behaviors of alamethicin channels (Keller et al., 1993; Bezrukov et al., 1998) and gramicidin channels (Lundbaek and Andersen, 1994; Lundbaek et al., 1997) are very sensitive to the spontaneous curvature of the lipid used for planar membrane formation.

The influence of membrane lipid composition on the bulk SRE concentration needed for single channel activity is shown in Fig. 8. Channel formation in pure DOPE membranes requires ~15-fold higher antibiotic aqueous concen-

tration than in pure DOPC membranes. Thus, planar membranes prepared from DOPE are less sensitive to SRE suggesting that the work of channel formation is much higher in nonlamellar than in lamellar lipid membranes.

The concentration dependence in Fig. 8 shows a sharp transition between low (~2.5 μg/ml) antibiotic concentrations for DOPC-rich mixtures and high (~35 μg/ml) antibiotic concentrations for DOPE-rich mixtures. This behavior does not follow the elastic stress of lipid packing obtained from spontaneous curvature measurements with similar mixtures (Keller et al., 1993). A possible reason for this discrepancy is that the lipid-induced change in the SRE channel energetics is not dominated by integral membrane characteristics, contrary to what is observed for alamethicin and gramicidin channels. The SRE channel may be a sufficiently strong defect in the membrane structure (Lundbaek and Andersen, 1999) so that the local effects including lipid segregation may be important.

It is tempting to estimate the additional energy cost for the channel formation in a nonlamellar lipid using the data shown in Fig. 8. To do this, we make a rather strong assumption. Specifically, we assume that SRE partitioning between the aqueous phase and the lipid membrane does not depend on lipid spontaneous curvature, i.e., the antibiotic partition coefficient is the same for all the DOPE/DOPC mixtures. Because the probability of SRE channel formation is proportional to the sixth power of antibiotic concentration, the 15-fold difference in SRE concentration indicates an $\sim 10^7$-fold suppression of channel formation by the nonlamellar DOPE. Rationalizing this result within the framework of Eqs. 1 and 2, we obtain a 16 kT increase for the structural component U_{ch} in case of DOPE. This estimate seems to be realistic. Although much higher energies are believed to be involved in formation of different local lipid structures (e.g., Kuzmin et al., 2001), it should be noted that the lipidic pore geometry includes both regions of high negative and high positive curvature (Chernomordik et al., 1985). Therefore, it is reasonable to expect some degree of compensation at the DOPE/DOPC substitution discussed here.

Fig. 9 shows typical recordings of SRE-induced currents in DOPC (upper part) and DOPE (lower part) membranes. The SRE channels in both cases are seen as well-defined similar steps between different current levels. The channels in DOPE membranes, however, are more persistent. Analyses of current histograms did not show significant differences in single-channel conductance for uncharged (DOPC, DOPE, and DPhPC) membranes (data not shown).

Statistical analyses of dwell times in the open state showed longer times in DOPE membranes, but reliable quantification was difficult because of the fast flickering closures seen in Fig. 9. Power spectral analysis of these recordings gave *1/f*-type spectra, even when fragments for the analysis were carefully selected to exclude transitions between different numbers of channels. Noise spectra of this

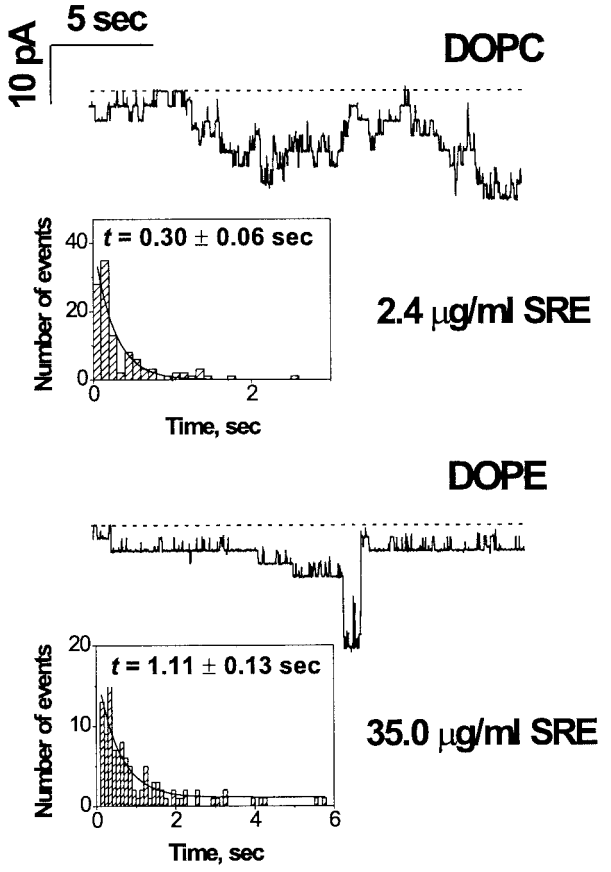

FIGURE 9 Typical current traces of the SRE channels in pure DOPC (*upper part*) and pure DOPE membranes (*lower part*). Time resolution was 10 ms. Insets show dwell time histograms for 110 single channels for both recordings. Crude single-exponential fit gives approximately fourfold longer dwell times for channels hosted by DOPE membranes. Applied voltage was −175 mV.

type are consequences of nonexponential, broad-time distributions. In our case the shape of the dwell time histograms was also dependent on the averaging time used in data preparation. Two histograms obtained at a 10-ms averaging represent typical results (Fig. 9, insets), showing that DOPE membranes yield longer living SRE channels. Therefore, although the nonlamellar lipid inhibits channel formation, the resulting channels are somewhat more persistent.

CONCLUSIONS

Using lipids of different charge and "molecular shape," we have studied ion channels produced in planar membranes by the lipopeptide antibiotic syringomycin E. Our results demonstrate that the channel functional properties are unusually sensitive to the host membrane lipids including their unprecedented involvement in channel gating. Specifically, we find:

1. Channel conductance is modified by lipid charge in a way that suggests that lipid headgroups are included into the structure of the ion-conducting pore.
2. Channel gating charge, which reflects changes in the average steady-state number of open channels in response to the applied voltage, is a strong function of membrane lipid composition. Manipulations of lipid charge (by either lipid species substitution or by proton titration) influence the absolute value and sign of the gating charge.
3. Nonlamellar lipids with the negative spontaneous curvature inhibit channel formation. Assuming that antibiotic partitioning between the aqueous phase and the lipid membrane is independent of the lipid spontaneous curvature, we estimate a 16-kT increase in the work of channel formation when DOPC is substituted with DOPE.

To rationalize these findings, we hypothesize that the syringomycin E channel is an asymmetric lipidic pore that is stabilized by antibiotic molecules. This crude sketch of the channel structure provides a qualitative explanation of our main experimental findings and gives a framework for future quantitative studies.

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