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Altering the Activity of Syringomycin E via the Membrane Dipole Potential

Olga S. Ostroumova,*,† Valery V. Malev,†,‡ Andrey N. Bessonov,†,§ Jon Y. Takemoto,|| and Ludmila V. Schagina†

Institute of Cytology of the Russian Academy of Sciences, St. Petersburg 194064, Russia, St. Petersburg State University, Petergof 198504, Russia, School of Engineering and Science, Jacobs University, Bremen, Germany, and Utah State University, Logan, Utah 84322-5305

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The membrane dipole potential is responsible for the modulation of numerous biological processes. It was previously shown (Ostroumova, O. S.; Kaulin, Y. A.; Gurnev, P. A.; Schagina, L. V. *Langmuir* **2007**, *23*, 6889−6892) that variations in the dipole potential lead to changes in the channel properties of the antifungal lipodepsipeptide syringomycin E (SRE). Here, data are presented demonstrating the effect of the membrane dipole potential on the channel-forming activity of SRE. A rise in the dipole potential is accompanied by both an increase in the minimum SRE concentration required for the detection of single channels at fixed voltage and a decrease in the steady-state number of open SRE channels at a given SRE concentration and voltage. These alterations are determined by several factors: gating charge, connected with translocations of lipid and SRE dipoles during channel formation, the bilayer−water solution partitioning of SRE, and the chemical work related to conformational changes during channel formation.

Introduction

Syringomycin E (SRE) belongs to a class of cyclic lipodepsipeptides produced by *Pseudomonas syringae* pv. *syringae* and plays an important role in the interactions of the bacteria with plants. SRE is highly toxic against fungi, yeast, and some bacteria.[1,2] Lipid bilayer studies have revealed that the mechanism of toxin action involves its insertion into target membranes followed by the formation of ion channels.[3−6] With one (cis) side addition, SRE forms ion channels of large and small conductance, and the "large" channels are clusters of the "small" (elementary) ones. It was shown that both the pore radius of small (elementary) channels included in clusters and their ionic selectivity are the same as those of elementary channels that are not included in clusters.[7] Also, the conductances of the clusters are multiples (and integer values) of the conductance of elementary channels.[4,7,8] Studies show that the elementary SRE channel is an asymmetric peptide−lipid pore having a conical shape with the wider trans opening formed from host lipid molecules.[9,10] These channels are preferentially anion-permeable, and their conductance and kinetics of opening (or closure) are strongly voltage-dependent.[3,4,9,11] The membrane conductance induced by SRE at fixed voltage increases with the sixth power of SRE concentration, suggesting that at least six toxin molecules are required for channel formation.[3,6,11] With negatively charged membranes bathed in 0.1 M NaCl at pH 6, the application of positive voltage opens the SRE channels. A reversal of the voltage sign leads to the closure of the channels. Charge screening with a 10-fold increase in the NaCl concentration reverses the sign of the opening/closing potential.[9]

The channel-forming activity of SRE (i.e., the ability to form pores in a membrane) can be expressed as the minimum concentration of SRE required for the observation of channel formation.[5] In addition, it can be characterized by a steady-state number of open channels under given experimental conditions.[12] Membrane composition (lipid charge, spontaneous curvature, presence of specific sphingolipids and sterols) and the presence of G- or F-actin or amphiphilic polymers on the trans side of the bilayer are known to influence SRE activity.[9,12−14]

The role of the membrane dipole potential, φ_d, is of particular interest because of its powerful impact on membrane permeability and lipid−protein interactions, including protein insertion, oligomerization, and function.[15−25] The value of φ_d is defined

* Corresponding author. E-mail: ostroumova@mail.cytspb.rssi.ru. Tel: +7 (812) 2972460. Fax: +7 (812) 2970341.

† Institute of Cytology of the Russian Academy of Sciences.

‡ St. Petersburg State University.

§ Jacobs University.

|| Utah State University.

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by the orientation of the electric dipoles of lipid head groups, fatty acid carbonyl groups, and membrane-adsorbed water.[26−28] The conductance of cationic channels formed from peptides such as gramicidin A and alamethicin decreases with increases in φ_d.[29−33] Luchian and Mereuta[33] also observed the alteration of alamethicin channel-forming activity with changing φ_d. In previous studies,[34,35] the effects of φ_d-modifying agents such as phloretin, RH 421, and 6-ketocholestanol on the properties of single SRE channels were studied. Increased φ_d led to multiple effects, namely, increases in SRE channel conductance and channel lifetime, a reduction in the number of synchronously functioning elementary channels, and changes in the sign and absolute value of the channel gating charge.

In the present study, the effects of dipole-modifying agents RH 421, phloretin, and 6-ketocholestanol on the channel-forming activity of SRE were investigated. A reduction of the membrane dipole potential induced by the addition of phloretin to the membrane-bathing solution led to a decrease in the minimum SRE concentration required for single channel formation and an increase in the steady-state number of open SRE channels at a given SRE concentration. An increase in φ_d by the addition of RH 421 to the membrane-bathing solution or the addition of 6-ketocholestanol to the membrane-forming solution yielded the opposite effects. Explanations for these alterations are considered in the framework of the proposed scheme of SRE channel formation. It is shown that the modulation of SRE channel formation by φ_d is determined by the changes in the gating charge of the channels, the membrane−water solution partitioning of SRE, and conformational changes occurring during the process of channel formation.

Experimental Section

All chemicals were reagent grade. Synthetic 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (PS) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PE) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone) and 6-ketocholestanol (5α-cholestan-3β-ol-6-one) were purchased from Sigma Chemical (St. Louis, MO), and RH 421 (*N*-(4-sulfobutyl)-4-(4-(4-(dipentylamino)phenyl)butadienyl) pyridinium, inner salt) was purchased from Molecular Probes (Eugene, OR). Water was distilled twice and deionized. NaCl solutions were buffered with 5 mM MOPS, pH 6.0. Syringomycin E (SRE) was purified as previously described.[36]

Virtually solvent-free membranes were prepared as described by Montal and Mueller.[37] The Teflon chamber was divided by a 15-μm-thick Teflon partition containing a round aperture of about 50 ÷ 100 μm diameter in two symmetrical halves with solution volumes of 1 cm^3. Squalene was used for aperture pretreatment. Membranes were made from an equimolar mixture of PS and PE. Syringomycin E was added to the aqueous phase on one (cis) side of the bilayer from water stock solutions (1 mg/mL). A pair of Ag−AgCl electrodes was used. "Virtual ground" was maintained on 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 the cis to trans side. Transmembrane currents were recorded with a custom-made amplifier and digitized using a pClamp-compatible board. Data acquisition was performed with a 5 kHz sampling frequency and low-pass filtering at 200 Hz. Data were analyzed using pClamp 9.0 (Axon Instruments) and Origin 7.0 (OriginLab). All experiments were performed at room temperature.

The dipole potential of the membrane was reduced by the two-side addition of phloretin to the bathing solution. The two-side addition of RH 421 to the membrane-bathing solution or of 6-ketocholestanol to the membrane-forming solution was used to increase the dipole membrane potential of phospholipid bilayers. A rough estimation of φ_d was made according to refs 19, 28, 34, and 38−42.

Taking into account the conductance multiplicity of the channel clusters with respect to elementary channels,[8] one can consider the steady-state transmembrane current at a given V (i.e., $I(V, \infty)$) to be the sum of currents passing through open elementary channels both included and not included in clusters of different multiplicity (i): $I(V, \infty) = I_1(V)\sum_{i=1}^{M(V)} iN_i(V, \infty)$. Here, $I_1(V)$ is the current passing through a single elementary channel;[35] $N_i(V, \infty)$ is the steady-state number of the ith channel type at the membrane surface with elementary channels ($i = 1$) and clusters that consist of $i \geq 2$ elementary channels operating simultaneously; and $M(V)$, being dependent on applied voltage, is the maximum number of channels included in a cluster.[8] Thus, the total number of open elementary SRE channels (both included and not included in clusters at given experimental conditions), $N_{op}(V, \infty)$, in bilayers of different dipole potential was obtained from the steady-state parts of current recordings as $I(V, \infty)/I_1(V)$.

Results and Discussion

The lowest SRE concentrations required for single-channel detection, $C(\varphi_d)$, at $V = 100$ mV were obtained in the presence and absence of agents that modify the dipole potential using membranes bathed in 0.1 and 1.0 M NaCl solutions. Table 1 shows that agents modifying the membrane dipole potential affect the channel-forming activity of SRE in planar lipid bilayers. The threshold concentration, $C(\varphi_d)$, required for single-channel recording at fixed voltage, V, is lower in the presence of phloretin (known to decrease φ_d)[27] than without a dipole modifier. On the contrary, in the presence of agents that increase the membrane dipole potential, such as RH 421 and 6-ketocholestanol,[23,28] $C(\varphi_d)$ is greater than that in the absence of these modifiers. Qualitatively the same effect of φ_d on $C(\varphi_d)$ was obtained with SRE-modified membranes bathed in 0.1 M NaCl (pH 6) (data not shown). Values of $C(\varphi_d)$ in the case with 0.1 M NaCl were about 2 times less than with 1 M NaCl. In spite of different $C(\varphi_d)$ values with

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Table 1. Threshold SRE Concentrations Required for the Observation of Single Channels in the Presence and Absence of Dipole Modifiers at $|V| = 100$ mV[a]

dipole modifier	φ_d, mV[b]	$C(\varphi_d)$, μM[c]
20 μM phloretin	120 ± 20	0.038 ± 0.016
no dipole modifier	230 ± 20	0.21 ± 0.10
5 μM RH 421	300 ± 40	0.39 ± 0.04
50 mol % 6-ketocholestanol	380 ± 20	0.47 ± 0.11

[a] Membranes were bathed in 1 M NaCl (pH 6). [b] The estimation of φ_d was made according to refs 19, 28, 34, and 38−42. [c] The values of $C(\varphi_d)$ were obtained using the identical diameter of the apertures in the Teflon film. The errors represent standard deviations of data obtained in experiments with three to seven bilayers.

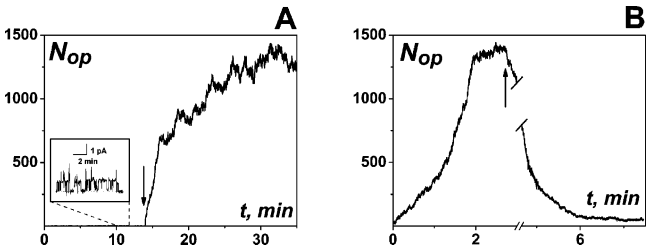

Figure 1. Effect of dipole-modifying agents on the total number of open elementary SRE channels. (A) The membrane was bathed in 0.1 M NaCl (pH 6), $V = 50$ mV. The addition of 20 μM phloretin to the bilayer-bathing solution is indicated by the arrow. (B) The membrane was bathed in 1 M NaCl (pH 6), $V = -50$ mV. The addition of 5 μM RH 421 to the bilayer-bathing solution is indicated by the arrow.

these two salt concentrations, the ratios of SRE concentrations in the presence and absence of dipole modifier were practically the same, probably as a result of the fact that φ_d does not depend on the salt concentration of the membrane-bathing solution.[39] Hence, one can conclude that the diminution of φ_d increases the channel-forming activity of SRE.

To understand the nature of the observed effect, a comparison was made of the steady-state total numbers of open elementary SRE channels, $N_{op}(V, \infty)$, in the bilayers at different dipole potentials (Figure 1). Figure 1A shows that the reduction of φ_d from 230 to 120 mV induced by the addition of 20 μM phloretin to the 0.1 M NaCl membrane-bathing solution leads to more than a 500-fold increase in $N_{op}(V, \infty)$ at 50 mV. Figure 1B shows that the increase in φ_d from 230 to 300 mV induced by the addition of 5 μM RH 421 to the 1 M NaCl solution decreases the N_{op} values by a factor of 20.

To ascertain the possible causes of the observed N_{op} changes (Figure 1), the following arguments are presented. The steady-state total number of open elementary SRE channels (both included and not included in clusters, see the Experimental Section) is

$$N_{op}(V, \infty) = \sum_{i=1}^{M(V)} iN_i(V, \infty) \quad (1)$$

By introducing such experimental parameters as the mean normalized number of synchronously functioning elementary channels in clusters, $M(V) = (\sum_{i=2}^{M(V)} iN_i)/(\sum_{i=2}^{M(V)} N_i)$, and the relative number of clusters, $S = (\sum_{i=2}^{M(V)} N_i)/(\sum_{i=1}^{M(V)} N_i)$, one can rewrite eq 1 as follows:

$$N_{op}(V, \infty) = N_1(V, \infty)\left[1 + \frac{m(V)S}{1 - S}\right] \quad (2)$$

It was shown[8,43] that m is a function of V and S is independent of V.

The model for SRE channel opening/closing that includes a common precursor for elementary SRE channels and their clusters was proposed by Malev and coauthors.[11] Under steady-state conditions, there must be equilibrium between precursors, $N_p(\infty)$, and elementary channels, $N_1(V, \infty)$ (i.e., the difference between the electrochemical potentials of these species is equal to zero: $\Delta\tilde{\mu} = 0$). From here, it follows that the natural logarithm of the equilibrium constant, $K_1(V)$, in its absolute value is nothing else than the work of channel formation expressed in heat units

$$kT \ln \frac{N_p(\infty)}{N_1(V, \infty)} = -kT \ln K_1(V) = \Delta U = \Delta U_{str} - eqV \quad (3)$$

where ΔU is the work of channel formation,[9,44] ΔU_{str} is a chemical or structural component related to conformational changes during the pore-formation process (in thermodynamics terms, ΔU_{str} is the change in the Gibbs standard energy at the transition from the closed state of the channel to its open state, i.e., it is the difference between standard values of the chemical potentials of the channel in the open vs closed states), q is the channel gating charge,[44] and eqV is the electrical work arising from displacements of charged and/or dipolar fragments during the same process (e is the electron charge). If the equilibrium relationship (eq 3) is taken into account, then eq 2 can be rewritten as

$$\ln N_{op}(V, \infty) = \ln N_p(\infty) + \ln\left[1 + \frac{m(V)S}{1 - S}\right] - \frac{\Delta U_{str}}{kT} + \frac{eqV}{kT} \quad (4)$$

$N_p(\infty)$ is determined by toxin concentration, C, and the partition coefficient, ρ, of SRE monomers between the lipid phase and water solution ($N_p(\infty) = K_p\rho^6C^6$, where K_p is the equilibrium constant between the SRE monomers and their aggregates on the membrane surface). $N_p(\infty)$ is assumed to be proportional to the sixth power of SRE concentration because the steady-state transmembrane current increased with the sixth power of SRE concentration.[3,6,11] Therefore, eq 4 may be rewritten as

$$\ln N_{op}(V, \infty) = \ln \rho' + 6 \ln C + \ln\left[1 + \frac{m(V)S}{1 - S}\right] - \frac{\Delta U_{str}}{kT} + \frac{eqV}{kT} \quad (5)$$

where $\rho' = K_p\rho^6$.

Experimental determinations of the steady-state number of open elementary SRE channels as a function of transmembrane voltage showed that $\ln N_{op}(V, \infty)$ was linearly dependent on eV/kT (Figure 2). Over the 50 mV range, the $m(V)$ dependence was weak[8] and did not significantly affect the $N_{op}(V, \infty)$ function. On the basis of eq 5, one can therefore conclude that the slope of the dependence of $\ln N_{op}$ on eV/kT is determined by q and the intersection (at $V = 0$) is determined by ρ', C, the cluster organization of SRE channels (values of m and S), and ΔU_{str}.

Earlier[34] it was reasonably assumed that the gating charge, q, is a sum ($q = q^{ch} + q^d$, where q^{ch} is related to translocations of charged species (SRE, lipids, and ions entering into a channel) and q^d is the gating charge component determined by translocations of lipid or/and SRE dipoles during channel opening).

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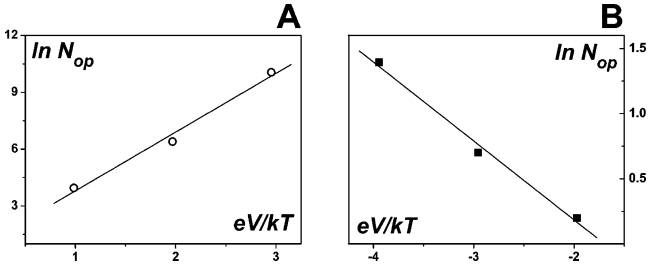

Figure 2. Logarithmic plots of the total number of open elementary SRE channels (N_{op}) in the presence of agents modifying the membrane dipole potential as a function of the applied voltage (V). Membrane-bathing solutions: (A) 20 μM phloretin + 0.1 M NaCl (pH 6); (B) 5 μM RH 421 + 1 M NaCl (pH 6).

Table 2. Relative Contributions of A and q to N_{op} for Membranes of Different Dipole Potentials[a]

	dipole modifier and membrane dipole potential			
	10 ÷ 20 μM phloretin $\varphi_{d1} = 100 \div 140$mV		5 ÷ 10 μM RH 421 $\varphi_{d1} = 280 \div 370$mV	
	0.1 M NaCl	1 M NaCl	0.1 M NaCl	1 M NaCl
$\exp[A(\varphi_{d1}) - A(\varphi_{d2})]^b$	3.6 ± 1.5	3.0 ± 0.9	0.3 ± 0.2	0.3 ± 0.2
$\lvert q(\varphi_{d1})\rvert - \lvert q(\varphi_{d2})\rvert^b$	2.2 ± 0.4	2.1 ± 0.6	-0.5 ± 0.2	-0.3 ± 0.2

[a] The errors represent standard deviations of data obtained in experiments with five to eight bilayers. [b] $\varphi_{d2} = (230 \pm 20)$ mV (no dipole modifiers).

Taking into account the dependence of N_{op} on φ_d (Figure 1) and neglecting the m dependence on V, eq 5 can be rewritten as follows

$$\ln N_{op}(V, \varphi_d, \infty) = A(\varphi_d) + \frac{q(\varphi_d)eV}{kT} \qquad (6)$$

where $A(\varphi_d) = \ln \rho'(\varphi_d) + 6 \ln C + \ln[1 + m(\varphi_d)\, S(\varphi_d)/(1 - S(\varphi_d))] - \Delta U_{str}(\varphi_d)/kT$ and $q(\varphi_d) = q^{ch} + q^d(\varphi_d)$. Therefore, the ratio of the total number of open SRE channels at different dipole potentials, φ_{d1} and φ_{d2}, is equal to

$$\frac{N_{op}(\varphi_{d1})}{N_{op}(\varphi_{d2})} = \exp[A(\varphi_{d1}) - A(\varphi_{d2})] \exp\left\{\frac{[\lvert q(\varphi_{d1})\rvert - \lvert q(\varphi_{d2})\rvert]e\lvert V\rvert}{kT}\right\} \qquad (7)$$

Here the absolute values of gating charge and transmembrane voltage are used because $N_{op}(V, \infty)$ depends on the product of these parameters, and the sign change of the potential that opens SRE channels in the presence of the dipole modifier is accompanied by the corresponding sign change of the gating charge.[34] This presents an opportunity to compare $N_{op}(V, \infty)$ at the same absolute value of transmembrane voltage with and without dipole modifying agents regardless of the sign of V that opens SRE channels. The present experimental results allow one to discriminate between the contributions to the total number of open elementary SRE channels of different factors that include voltage-independent factors (bilayer−water solution partitioning of SRE, cluster organization, and chemical constituents of channel formation) and the gating charge that determines voltage sensitivity (Table 2). The mean values of q and A in the absence and in the presence of dipole modifiers were used. It should be pointed out that values of $A(\varphi_{d1})$ and $A(\varphi_{d2})$ must be evaluated together in the same experiment with the same membrane at a given SRE concentration because A is a function of C. In that case, the difference $A(\varphi_{d1}) - A(\varphi_{d2})$ should not depend on C, which makes it possible to average the obtained $[A(\varphi_{d1}) - A(\varphi_{d2})]$ data over experiments with different C values. Table 2 shows that the dipole potential modified the SRE channel-forming activity as a result of its influence on both $A(\varphi_d)$ and $q(\varphi_d)$. As seen in Table 2, the increase in φ_d was accompanied by the reduction of A and $|q|$.

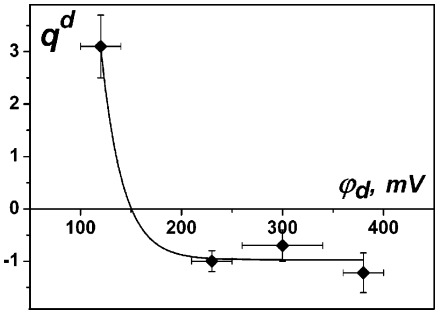

Figure 3. Gating charge of the elementary SRE channel related to translocations of lipid and toxin dipoles (q^d) as a function of the membrane dipole potential (φ_d). The membranes were bathed in 1 M NaCl (pH 6).

The reduction of A induced by the increase in φ_d may be result of decreasing the parameters ρ', m, and S or by increasing the structural component, ΔU_{str}. In a recent publication,[35] we reported that an increase in φ_d from 120 to 300 mV results in a decrease in the mean number of synchronously operating elementary channels in the clusters, m, from 9 to 6. The relative number of SRE clusters, S, practically does not depend on φ_d. In the absence of dipole modifiers and in the presence of 20 μM phloretin in the 1 M NaCl solution bathing the membranes, S is equal to 0.27 ± 0.04 and 0.22 ± 0.05, respectively. Corresponding calculations show that the contribution of the component depending on m and S to observed changes of $\exp[A(\varphi_{d1}) - A(\varphi_{d2})]$ in bilayers of different φ_d (Table 2) does not exceed 15%. Thus reduction of A caused by the increase in φ_d resulted from decreasing ρ' and/or increasing ΔU_{str}. On the basis of eq 7, it is concluded that the contribution of the voltage-dependent component $\exp\{[|q(\varphi_{d1})| - |q(\varphi_{d2})|]e|V|/kT\}$ to N_{op} increases with increasing $|V|$. Data presented in Table 2 show that at $V > 100$ mV the input of the above component is larger than that of the voltage-independent component ($\exp[A(\varphi_{d1}) - A(\varphi_{d2})]$).

According to ref 39, the membrane dipole potential is the same for membranes bathed in solutions of different ionic strength. Therefore, the differences in the $|q(\varphi_{d1})| - |q(\varphi_{d2})|$ values in the membranes bathed in 0.1 versus 1 M NaCl (Table 2) are not influenced by charge screening. Instead, the absolute values of $q(\varphi_d)$ in the membranes bathed in 0.1 and 1 M NaCl (pH 6) are different as a result of charged components. Figure 3 shows the dependence of q^d on φ_d in membranes bathed in 1 M NaCl solution (i.e., with charge screening to exclude q^{ch} from the total gating charge $q(\varphi_d) = q^{ch} + q^d(\varphi_d)$). The sign change of q^d from positive to negative values with increasing membrane dipole potential may be due to orientation differences of lipid dipoles in the SRE channel in the absence and presence of phloretin. However, sign differences of the gating charges that are related to the dipole orientation of channel lipids (q^d_{lipids}) and SRE molecules (q^d_{sre}) are also possible because the dipole component of the channel gating charge is the algebraic sum $q^d = q^d_{lipids} + q^d_{sre}$. The SRE positive pole should be oriented to the hydrocarbon region of the bilayer, and hence the reduction

of φ_d will facilitate SRE insertion into the cis monolayer and increased membrane−water solution partitioning.

Using similar methods, Luchian and Mereuta[33] investigated the channel-forming activity of alamethicin in the presence of dipole modifiers, such as phlorizin and 6-ketocholestanol. Similarly to phloretin, phlorizin decreases the membrane dipole potential. Because alamethicin monomers have positively charged N termini, a decrease in the membrane dipole potential is accompanied by a reduction of the energy barrier for the interfacial accumulation of alamethicin monomers on the cis side of the membrane. The authors consider that the lack of voltage dependence of the phlorizin-mediated increase in alamethicin activity is evidence of increased lipid membrane−water solution partitioning of alamethicin monomers. In the context of the present work, it is also possible that there is a contribution of the chemical component related to conformational changes during alamethicin pore formation to the peptide activity in membranes of different dipole potentials.

In summary, a detailed investigation of the mechanism of modulation of SRE activity by φ_d is presented. On the basis of the experimental data, a theoretical approach is given that allows discrimination between the influences of different factors on N_{op} values. It is concluded that the channel-forming activity of SRE induced by the membrane dipole potential is complex and connected to the gating charge due to translocations of dipoles, the bilayer−water solution partitioning of SRE, and the structural component of channel formation.

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