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Effective gating charge of ion channels induced by toxin syringomycin E in lipid bilayers

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Abstract

To elucidate the voltage gating of syringomycin E (SRE) ion channels in lipid bilayers, the effective gating charge q was measured under different conditions. It was shown that q and its sign are dependent on membrane surface charge, dipole potential, and the outer potential ($\Delta\phi$). The q values were positive for charged bilayers and negative for uncharged bilayers bathed in the same 0.1 M NaCl solutions. Effects of dipole modifying agents on the gating properties of SRE channels were measured. In uncharged bilayers, addition of phloretin resulted in an increase of q values. For charged bilayers, the presence of RH-421 or 6-ketocholestanol leads to the reverse in the sign of q from positive to negative. The q values were potential-dependent at higher negative voltages with charged membranes bathed in solutions with high salt concentrations. It is concluded that lipid molecules participating in the SRE channel structure contribute to channel formation work due to Coulomb and dipolar interactions with the electric field applied to a membrane. The potential dependence of q is explained by interactions of charged and uncharged lipids with SRE molecules in the channels.

Keywords: Ion channels; Lipid membranes; Channel voltage gating

1. Introduction

This work is a continuation of investigations of the ion channels formed in planar lipid membranes by the phytotoxin syringomycin E (SRE). Our goal is to elucidate the role of charged and dipolar components of lipid bilayers in the voltage gating of these channels. SRE is a cyclic lipodepsinonapeptide produced by the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae*. The SRE molecule consists of the polar peptide head and a hydrophobic 3-hydroxydodecanoic fatty acid tail. The polar head is a macrocyclic lactone ring containing nine amino acid residues, three positively charged, and one negatively charged [1–3]. SRE is toxic to many yeast and fungal species [4–6]. Based on comparative studies of SRE activity and of its analogs in living cells and lipid bilayer membranes, the cytotoxic effect has been proposed to result from a pore-forming activity in the plasma membrane [7,8]. The channels formed by SRE in bilayer lipid membranes are preferentially permeable to anions [9–14]. Their lumen radius is about 1 nm [14–16], and two types of the channels, ‘‘small’’ and ‘‘large’’, differing six to seven times in their conductance, have been observed [13,14]. The kinetics of channel opening (and closing) is strongly voltage dependent, and the channel current–voltage curves are asymmetric and nonlinear in the voltage [8,12,13,17,18]. Also, the sign of the potentials that open and close the SRE channels depends on membrane lipid composition, the pH and salt concentration of the bathing solution [18]. The above findings have made it possible to consider SRE channels as asymmetrical lipid pores stabilized by several toxin molecules situated near the *cis*-mouth of the channel (the side of SRE addition to the membrane). The inclusion of lipids into the channel structure leads to the electrostatic involvement of both their charges and dipoles in the channel formation. Calculations show that the energy contribution

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from dipolar moments of lipid molecules might exceed kT several times, i.e. the effect related to the dipole nature of lipid molecules should be observed under experimental conditions [18]. Indeed, this contribution was observed when using the dipole modifying agent, phloretin [19].

The opening/closure of voltage-dependent ion channels is usually considered in terms of an energy change, ΔU, at a transition from an open to closed state of a channel. ΔU is given by the equation:

$$\Delta U = \Delta U_{ch} - eq\Delta\phi - \alpha\Delta\phi^2, \quad (1)$$

where $q = q_c + q_d$ and $\Delta\phi$ is the transmembrane potential. Here, U_{ch} is the chemical component of ΔU; $eq\Delta\phi$, the component from displacements of charged and/or dipolar molecules in the electric field applied to the membrane; e, the electron charge; $\alpha\Delta\phi^2$ is the component included in the ΔU to account for possible electrostriction. The dimensionless parameter q, reflecting a possible difference in positions of charged and/or dipolar molecules in open and closed states of the channel, was accepted to call an "effective gating charge" or simply "gating charge"; q_c and q_d are charged and dipole components of gating charge q, correspondingly. The mean number of open channels under steady-state conditions, N_{ch}, is related to ΔU by the equation:

$$N_{ch} = N_t/[1 + \exp(\Delta U/kT)], \quad (2)$$

where N_t is the total number of both closed and open channels [20–22] (see also Ref. [18]). Excluding some special cases that will be indicated further on in our experiments with SRE modified bilayers, we have not seen the saturation in N_{ch} as a function of $\Delta\phi$ predicted by Eqs. (1) and (2). This means that the inequality $\exp\Delta U/kT \gg 1$ is fulfilled under the conditions of our experiments, and therefore

$$N_{ch} \propto \exp\{(eq\Delta\phi + \alpha\Delta\phi^2)/kT\}. \quad (3)$$

Thus, the gating charge q and the "electrostriction parameter" α can be obtained from the dependence of the steady state number of open channels on the transmembrane potential $\Delta\phi$. That number was found to be exponential in the range of $\pm$ 120 mV [12,18,19], excluding special cases mentioned below. This means that the work of channel formation is linear in potential $\Delta\phi$, and the electrostriction force responsible for the $\alpha\Delta\phi^2$ component does not significantly affect the channel opening. This allows determinations of q values by using the expression:

$$q \cong d(\ln N_{ch})/d(e\Delta\phi/kT), \quad (4)$$

which takes into account the insignificance of the electrostriction constituent $\alpha\Delta\phi^2$ and, hence, independence of q on the applied voltage. Special cases of voltage-dependent q will be discussed in this paper.

For membranes formed from an equimolar mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), the SRE channel gating charge determined as described above is close to unity at 0.1 M NaCl concentration. With neutral lipids (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine, DPhPC) in 0.1 M NaCl, the gating charge is negative [18,19]. These results show that manipulations of lipid charge affect both the absolute value and the sign of the gating charge. Thus, measurements of q that characterize sensitivity of the steady state number of open SRE channels to the applied voltage can provide important information on channel energetics and, particularly, on the role of different components of the channel structure in its formation.

In this paper, we use these measurements of q to show that lipid charges and dipoles affect the voltage gating properties of SRE channels. It will be demonstrated that the sign of q can be inverted not only by changes in the membrane system composition, but also by increasing the voltage applied to charged membranes bathed in an electrolyte solution of high ionic strength.

2. 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 (Pelham, AL). Poly-L-lysine hydrobromide (PL) with molecular weight of 30 000 (DP = 140) and poly-L-glutamic acid sodium salt (PG) with molecular weight of 27 500 (DP = 160) were purchased from Sigma (St. Louis, MO). All the salts used to prepare electrolyte solutions were of reagent grade (Sigma). The solutions were buffered with 5 mM MOPS/NaOH to pH 6.0. Water was deionized and double distilled. Syringomycin E was purified as described previously [23]. Phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone), 6-ketocholestanol (5α-cholestan-3β-ol-6-one) were purchased from Sigma and RH-421 (*N*-(4-sulfobutyl)-4-(4-(4-(dipentylamino)phenyl)butadienyl) pyridinium inner salt) from Molecular Probes (Eugene, OR).

The solvent-free membranes were prepared as described by Montal and Mueller [24]. The membrane-forming solutions were DOPS, DPhPC, DOPC, and mixtures of DOPS, DOPE, and 6-ketocholestanol in hexane. Two symmetrical halves of a Teflon chamber with solution volumes of 1.5 cm^3 were separated with a 15-μm-thick Teflon partition containing a round aperture of about 100-μm diameter. Hexadecane in *n*-hexane (1:10, v/v) was applied for aperture pretreatment. A pair of Ag–AgCl electrodes was used to maintain the membrane potential and to detect ion currents. The term 'positive voltage' means that the *cis*-side compartment (the side of SRE addition) is positive with respect to the *trans*-side.

SRE was added to the aqueous phase after bilayer formation from water stock solutions. The total SRE concentration in the membrane-bathing solution did not exceed 0.03 mM. Phloretin and RH-421 were added to the aqueous phase before bilayer formation from the ethanol stock solution. 6-Ketocholestanol was added to the membrane-forming solution. PL or PG was added to the membrane-bathing solution after bilayer formation to a final concentration equivalent to 10 μM lysine or glutamate. All the experiments were performed at room temperature. The methods used for membrane preparation and single-channel data analyses were described previously [25].

The mean values of the current, I, through single channels were obtained from current histograms. For each current level, a current amplitude histogram fitted a Gaussian distribution, using the "Origin" software (Microcal Software, Inc; Northampton, MA). Conductance–voltage curves are presented as integral channel conductance $G = I/\Delta\phi$ as a function of the membrane potential, $\Delta\phi$. 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 provided the total number of the open small channels, N_{ch}, which was then used to obtain the effective gating charge, q (for details, see Ref. [18]).

Membrane stability during the long-term experiments necessary for measuring q values was much better when using DOPS and mixtures of DOPS and DOPE bilayers that have a negative charge at pH 6. In the case of the neutral DOPE membranes, only single channels were observed after addition of SRE up to 100 μM in 0.1 M NaCl, pH 6 [18]. That was the reason why DOPC and DPhPC lipids were used to obtain the multichannel current data in the case of uncharged membranes.

3. Results and discussion

The q values obtained for different ratios of charged (DOPS) vs. uncharged (DOPE, DPhPC, DOPC) lipids in membranes bathed in 0.1 M NaCl are presented in Fig. 1. The q value can be seen to strongly depend on membrane charge. With an increase in the fraction of DOPS, q increased from -1 (no DOPS) to 1.5 (DOPS only), and it was zero at approximately 20 molar% DOPS. The most dramatic change in q occurred within the range of 10–30 molar% of DOPS. Even beyond this region, the sign of q can be inverted by introducing agents that modify the membrane dipole potential. Thus, RH-421 and 6-ketocholestanol are known to increase the membrane dipole potential [26–28], while phloretin decreases it [28–30]. Their effect on the gating charge of SRE channels is shown in Figs. 2 and 3. Addition of RH-421 to the bathing solution

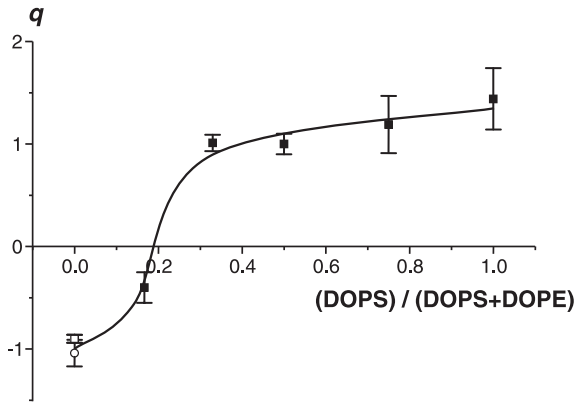

Fig. 1. Dependence of the effective gating charge of SRE channels q on the fraction of charged lipid (DOPS) in the membrane-forming solution (■). At 0 molar% of DOPS, membranes were formed from DPhPC (○) or from DOPC (□). Membranes were bathed by 0.1 M NaCl solution (pH 6).

(0.1 M NaCl) or substitution of uncharged lipid (DOPE) in the membrane-forming solution for 6-ketocholestanol produced negative values of q, although q was positive and equal to 1 in the absence of these substances (Fig. 2). The opposite effect occurred with incorporation of phloretin into uncharged bilayers (Fig. 3). The effect of the dipole modifying agents on q values demonstrates a significant contribution of dipolar molecules to the channel formation work. At the same time, these data show that charged and dipolar molecules included in a channel provide comparable contributions to the gating charge q. These contributions are opposite in sign and thus account for the observed sign reversal in q values with the dipole modifying agents.

The resultant contribution of the charge q_c and dipolar q_d constituents to gating charge q are easily shifted by changing the composition of the membrane-bathing solution. As reported previously [18], the sign of q is negative (about -1) when charged bilayers of an equimolar mixture of DOPS and DOPE are bathed in 2.5 M NaCl. However, for the same bilayers, the q value is close to 1 in 0.1 M NaCl. This phenomenon is due to screening of the lipid and SRE charges on the channel wall with ions present in the aqueous interior of a channel. In parallel to charged lipids and SRE molecules, ions of opposite charges enter the channel interior to partly compensate charges of the channel-forming molecules. The charge q_c resulting from displacements of all the molecules included in a channel is lower when the bathing electrolyte concentration is higher. If q_c goes below q_d, the q value changes sign.

The q values measured at 1 M NaCl in the voltage range of 0 to -100 mV were equal to -0.84 ± 0.30 (S.D.) regardless of DOPS concentration in the membrane-forming solution. However, unlike the case with 0.1 M NaCl, q remained at a constant negative value only at relatively low negative $\Delta\phi$ and then reduced its absolute value below -100 mV, so that it became positive at higher negative $\Delta\phi$ (Fig. 4). Fig. 4 insert shows dependence of N_{ch} on $\Delta\phi$. The

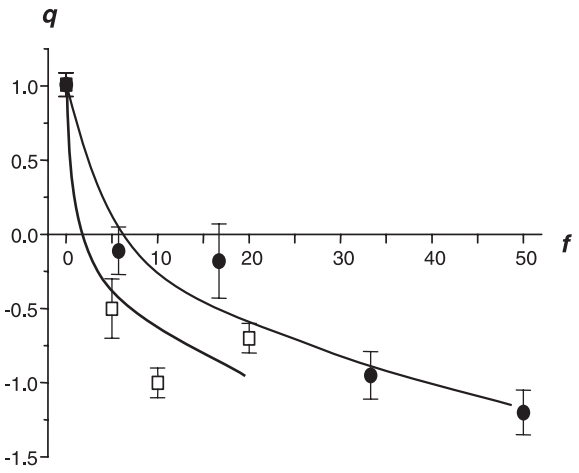

Fig. 2. Effective gating charge of SRE channels, q, as a function of modifying addenda concentrations. The gating charge q was measured at absolute values of potential $\Delta\phi$ less than 100 mV. Open squares (□) and solid circles (●) correspond to data obtained in the presence of RH-421 in the membrane-bathing solution and 6-ketocholestanol in the membrane-forming solution, respectively. For the first case, abscissa f shows concentrations of RH-421 (μM), but gives the molar content (%) of 6-ketocholestanol, which replaced DOPE in DOPE + DOPS (30%) bilayers for the second case. The membrane-bathing solution was 0.1 M NaCl (pH 6) for both cases.

number of open channels was found to decrease with increasing absolute value of $\Delta\phi$. The qualitatively similar behavior of $q(\Delta\phi)$ takes place for charged membranes modified by RH-421 or 6-ketcholestanol, but the bathing electrolyte concentration in such systems is 0.1 M. The existence of the $q(\Delta\phi)$ dependence in the cases of RH-421 and 6-ketcholestanol is the reason for the use of the range of potentials ($\Delta\phi \leq |100\ \text{mV}|$) applied for the q value determinations presented in Fig. 2.

It is emphasized that the $q(\Delta\phi)$ dependence was observed only for charged membranes bathed in solutions of a sufficiently high ionic strength. The electrostriction constituent proportional to $\Delta\phi^2$ (see Eq. (1)) cannot be the

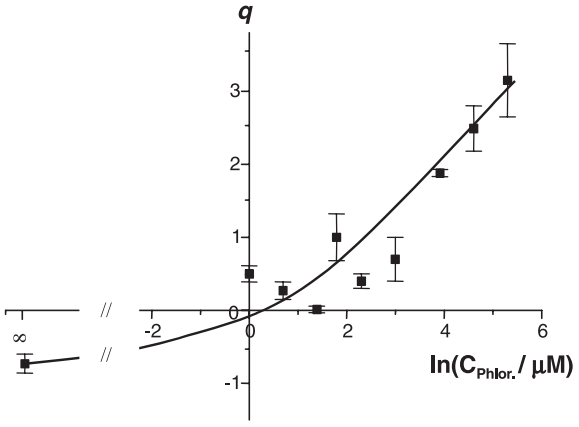

Fig. 3. Dependence of the effective gating charge q of SRE channels on the concentration of phloretin in the membrane-bathing solution, containing 0.1 M NaCl (pH 6). Membranes were prepared from DPhPC.

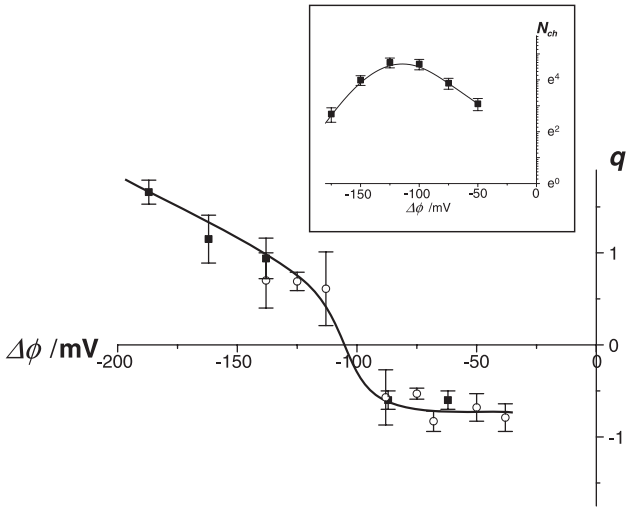

Fig. 4. Potential dependence of the q values of SRE channels in membranes formed from DOPS (■) and from equimolar mixtures of DOPS and DOPE (○). The membrane-bathing solution was 1 M NaCl (pH 6). Insert: Dependence of the number of open SRE channels on the transmembrane potential. Membranes were prepared from DOPS and bathed in 1 M NaCl (pH 6).

source of this dependence. This conclusion is confirmed by the fact that electrostriction usually facilitates pore formation, i.e. electrostriction parameter $\alpha>0$ [31]. If electrostriction was responsible for the discussed effect, the later would be observed for neutral bilayers. However, this is not the case. Thus, the dependence $q(\Delta\phi)$ only resulted from a specific effect of high negative potentials on the components of SRE channels. Since the dependence is specific to charged membranes at high salt concentration, one can suggest that the phenomenon is related to the effect of the channel interior electric field on adsorption of Na^+ ions on the channel wall.

It is relevant that the absorption constant of Na^+ ions for the surface of DOPS liposomes is about 0.6–1 M^{-1} [32]—close to the concentration at which the q dependence described above was observed. Although negligible at low $\Delta\phi$, the electric field effect on this absorption should be nonlinearly dependent on the applied voltage at high transmembrane potentials. Thus, a potential-dependent absorption of Na^+ ions is possible, and what remains open is a concrete explanation of how the absorption of Na^+ ions affects the channel formation.

Interactions between SRE molecules and neighboring lipids contribute substantially to the chemical constituent (ΔU_{ch}) energy change ΔU at opening/closure of SRE channels. In particular, the Coulomb binding energy of molecules of opposite charges (SRE species and charged lipids) is an important factor in these interactions. It is suggested that the energy of such interactions significantly decreases with increased Na^+ ion adsorption, as adsorbed cations neutralize the membrane charge. Since the cation adsorption is potential-dependent, the fraction of charged lipids included in a channel will also change as a function of

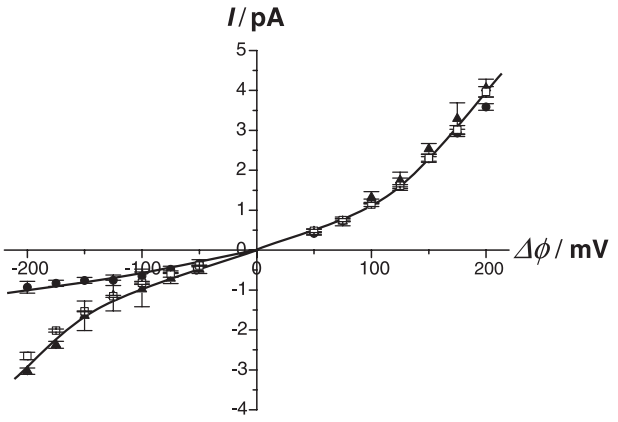

Fig. 5. Current–voltage curves of single SRE channels in DPhPC membranes in the absence of PG (□) and in the presence of PG (10 μM) on the *cis*-side (●) and on the *trans*-side (▲). Membranes were bathed by 0.1 M NaCl (pH 6) solution.

outer voltage. Also, interactions of charged and neutralized lipids with SRE molecules of the channel will be different, and the number of open channels N_{ch} might decrease with an increase of the negative value of $\Delta\phi$ (see Fig. 4, insert). In other words, q might change its sign although the dipolar constituent facilitates the channel opening at negative $\Delta\phi$. Thus, changes in cation adsorption, which are induced by the applied voltage, are assumed to underlie the phenomenon in question. This suggestion is supported by the observations described below.

Changes of salt concentrations on the *trans*-side of the membrane (from 0.1 to 1 M NaCl) after its modification by SRE in symmetrical 0.1 M NaCl solutions lead to more than a 100-fold decrease in the number of open SRE channels. This decrease cannot be ascribed to the intramembrane jump of potentials, which arise in such asymmetrical systems. This jump would not exceed −40 mV ($t_{Cl} \approx 0.7$ under these conditions [13]) and, hence, its effect would only lead to a five-fold decrease ($q \cong 1$). No decrease in number of open channels was observed when 1 M NaCl was added to the *cis*-side of the membrane.

The asymmetric addition of positively charged PL (10 μM) to the *trans*-compartment of the charged membrane system (equimolar mixture of DOPS and DOPE) and 0.1 M NaCl bathing solution decreased the conductance in ways that resemble a salt gradient, i.e. the number of open channels, N_{ch}, decreased about 50-fold. The number N_{ch}, as well as the current–voltage characteristics of SRE channels, did

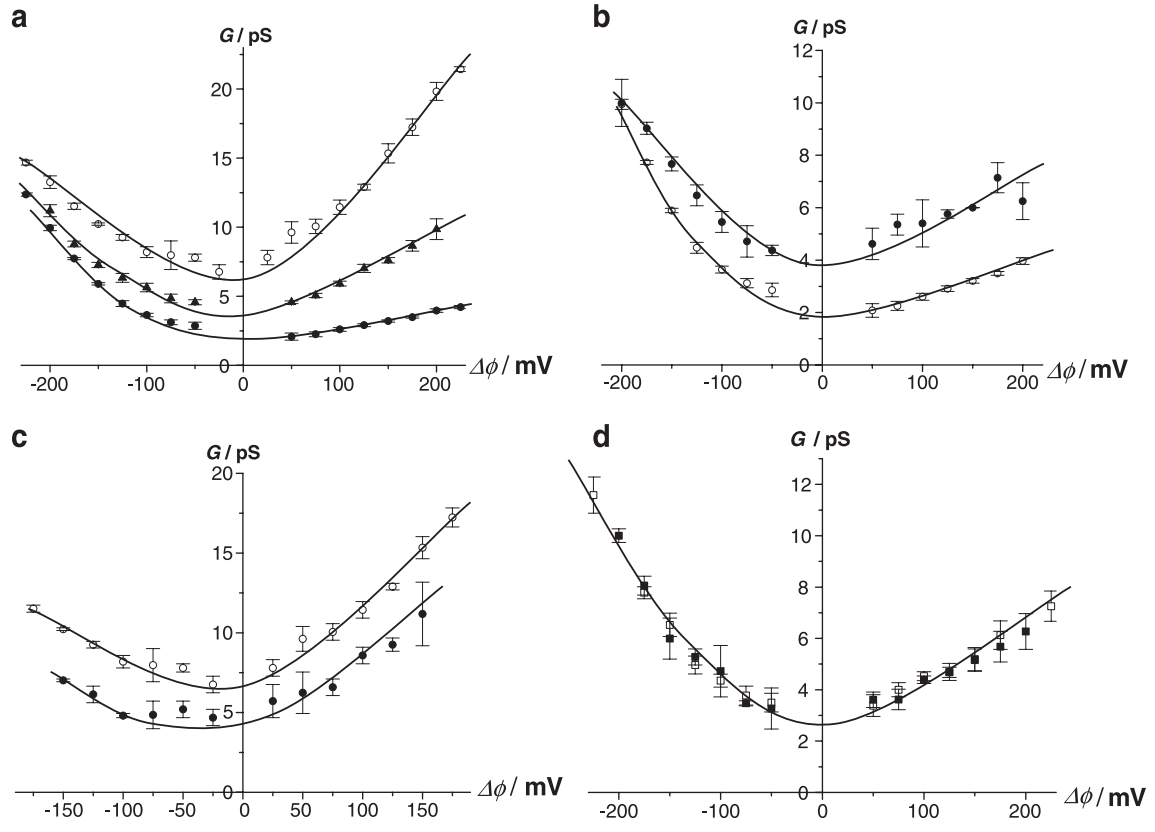

Fig. 6. Conductance–voltage curves of single SRE channels in membranes of various composition(s). (a) Membranes contained different fractions of charged (DOPS) and uncharged (DPhPC) lipids. Concentrations of DOPS in molar% were 0 (○), 20 (▲) and 100 (●). (b) Membranes from equimolar mixture of DOPS and DOPE in the absence (○) and the presence (●) of 10 μM of RH-421 in the membrane-bathing solution. (c) DPhPC membranes in the absence (○) and the presence (●) of 20 μM of phloretin in the membrane-bathing solution. (d) Membranes in the absence (DOPS/DOPE, 1:2, M/M) (□) and the presence (DOPS/DOPE/6-ketocholestanol, 1:1:1, M/M/M) (■) of 6-ketocholestanol. Results presented in these figures were obtained for membranes bathed by 0.1 M NaCl (pH 6) solution.

not change after PL addition to the *cis*-compartment. The simplest explanation for these results, as well as of the salt gradient effect, is that specific interactions occurring between *trans*-side DOPS and SRE molecules significantly decrease due to neutralization of the DOPS charge with increased salt concentration or addition of PL.

Within the scope of the above explanation, the voltage dependence of q should increase, as the part of the outer potential $\Delta\phi$ on the *trans*-side of the channel increases. Particularly, if this part is negligible, the $q(\Delta\phi)$ dependence will be absent. Hence, an estimate of the electric potential distribution along the channel length is needed to check the validity of the explanation in question. This problem was partly solved by studying the effect of PG anions on the SRE channel properties. Typical current–voltage curves obtained for single SRE channels in the absence and presence of these anions (10 μM, *cis*-side addition to DPhPC membranes bathed in 0.1 M NaCl) are presented in Fig. 5. These curves diverged only at high negative voltages. Addition of PG anions to the *trans*-compartment did not change the channel conductance at all studied potentials (− 200–200 mV). This is not surprising since the size of a PG anion is too large to penetrate the channel on the *trans*-side. These findings show, first, that the PG anions interact with the *cis*-mouths of the channels and, second, that their binding is insignificant for anion transport through the channels at low potentials, but hinders it at high negative $\Delta\phi$. Regardless of the mechanisms of this inhibiting effect, it indicates the existence of the electric potential drop near the *cis*-mouths of SRE channels and a significant variation of that drop with change of the outer potential. It is reasonable to think that, in addition to the potential drop within the *cis*-mouth of the channels, a significant part of the total applied $\Delta\phi$ is distributed within the *trans*-side of the channels. If so, the phenomenon of potential-dependent $q(\Delta\phi)$ must be real.

Pronounced changes in the single-channel conductance accompanied the above variations in the chemical composition of the membrane system. As seen from Fig. 6a, the asymmetry of the conductance–voltage curves changed markedly with variation of the charged lipid fraction. The $G(\Delta\phi)$ curves were asymmetric within the limits of completely charged or uncharged membranes, but became symmetric at about 20 molar% of DOPS. Consistent with the effects of RH-421 or phloretin addition on q, changes in the single-channel conductance were observed with these agents. RH-421 increased the channel conductance, while phloretin decreased it (Fig. 6b,c). At the same time, no SRE channel conductance changes were observed when 6-keto-cholestanol was substituted for the uncharged (DOPE) component of the bilayer (Fig. 6d). It is suggested that the observed sensitivity of the channel conductance to the presence of dipole modifying agents is mainly due to the changes in the electric field within the channel interior, which accompany variations of the membrane chemical composition. Also, structural changes in the channel lumen or of the channel shape cannot be ruled out.

4. Conclusions

1. The effective gating charge of the SRE channels is highly sensitive to charge and dipolar components of the membrane in support of the participation of membrane constituents in the structure and function of these channels.
2. The contributions of the charged and dipolar constituents to the effective gating charge of SRE channels are comparable in absolute values but opposite in sign.
3. The potential dependent channel gating charge values result from specific interactions between charged lipids and SRE molecules in the channel structure as well as adsorption of Na^+ ions on the channel wall.

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