Membrane Surface-Charge Titration Probed by Gramicidin A Channel Conductance

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ABSTRACT   We manipulate lipid bilayer surface charge and gauge its influence on gramicidin A channel conductance by two strategies: titration of the lipid charge through bulk solution pH and dilution of a charged lipid by neutral. Using diphytanoyl phosphatidylserine (PS) bilayers with CsCl aqueous solutions, we show that the effects of lipid charge titration on channel conductance are masked 1) by conductance saturation with Cs^+ ions in the neutral pH range and 2) by increased proton concentration when the bathing solution pH is less than 3. A smeared charge model permits us to separate different contributions to the channel conductance and to introduce a new method for "bilayer pKa" determination. We use the Gouy-Chapman expression for the charged surface potential to obtain equilibria of protons and cations with lipid charges. To calculate cation concentration at the channel mouth, we compare different models for the ion distribution, exact and linearized forms of the planar Poisson-Boltzmann equation, as well as the construction of a "Gibbs dividing surface" between salt bath and charged membrane. All approximations yield the intrinsic pK_a^{in} of PS lipid in 0.1 M CsCl to be in the range 2.5–3.0. By diluting PS surface charge at a fixed pH with admixed neutral diphytanoyl phosphatidylcholine (PC), we obtain a conductance decrease in magnitude greater than expected from the electrostatic model. This observation is in accord with the different conductance saturation values for PS and PC lipids reported earlier (Apell et al. 1979, *Biochim. Biophys. Acta.* 552:369–378) and verified in the present work for solvent-free membranes. In addition to electrostatic effects of surface charge, gramicidin A channel conductance is also influenced by lipid-dependent structural factors.

INTRODUCTION

The phospholipid/water interface plays a crucial role in membrane transport and membrane-bound enzyme regulation. The charge of lipid polar groups and ion absorption create electrostatic potentials that modulate the concentration of protons, metal and halide ions, and other charged substrates. Understanding these regulatory processes requires knowledge of lipid polar group structure, organization, and state of ionization. The ionization of lipids at a given pH and ion concentration in the bulk depends on intrinsic pK_a, ion-lipid binding constants, molecular packing, and temperature (e.g., see review by Tocanne and Teissié, 1990).

Some 4–20% of the lipids in membranes of many cells and organelles bear a net negative charge (White, 1973). The source of the negative charge is mainly phosphatidylserine (PS), the major charged lipid in the bilayer component of the mammalian cell membrane. To determine the contribution of this lipid to the total membrane charge, its intrinsic pK_a^{in} should be known. Measurements of apparent pK_a values have been made on vesicles (Tsui et al., 1986), dispersions (MacDonald et al., 1976; Cevc et al., 1981), monolayers (Ohki and Kurland, 1981; Moncelli et al.,

1994), and bilayers (Matinyan et al., 1985). The intrinsic pK_a^{in} reported for the carboxyl group (Tocanne and Teissié, 1990) spans a wide range from 2.62 (Tokutomi et al., 1980) to 3.6 (Tsui et al., 1986). These differences probably account for the fact that the observed pK_a of a charged group can be significantly altered by the local environment. The determination of intrinsic pK_a is sensitive to the membrane lipid density, curvature, hydrophobic content, lipid mixture composition, and, consequently, the technique used.

Because of their structural and electrical similarity, planar bilayer lipid membranes are well-established models of biological membranes. In particular, intrinsic pK_a^{in}'s of lipids found in experiments with planar lipid bilayers are biologically instructive.

Gramicidin A is an uncharged pentadecapeptide that forms highly selective cation channels across lipid bilayers (Koeppe and Andersen, 1996). Over the last three decades these channels have been extensively studied from many different points of view to include their structure, conformational dynamics, ion selectivity, water permeability, etc. The large amount of available information provides an excellent opportunity to use the gramicidin channel as a tool to study other biophysical systems that are not so well characterized. In particular, it can be used as a probe to access the membrane surface and to study the ionization state of the various ionizable phospholipid groups complementing other methods currently used.

There is solid experimental evidence that the electric conductance of ion channels can be influenced by the fixed charge of lipid polar headgroups. Apell et al. (1979) re-

ported much higher gramicidin A channel conductance in negatively charged membranes than in neutral membranes. Similar behavior was found for other channels embedded in charged membranes (Bell and Miller, 1984; Moczydlowski et al., 1985; Coronado and Affolter, 1986; Green and Andersen, 1991). This effect is particularly strong in low ionic strength electrolytes. As shown in Fig. 1, a simple qualitative explanation in terms of basic electrostatics can be invoked. According to the accepted model of the gramicidin channel (a 3-nm-long cylinder with a total diameter, including the peptide wall, of 1.2–2.6 nm; see table 2 in Woolf and Roux, 1996), the lipid molecules surround the pore with a distance between centers of their polar heads and the channel axis that is rather close to the Debye length ($\lambda_D \approx 1.0$ nm) of 0.1 M salt solutions. Negative charge on the membrane drives away coions and increases the concentration of oppositely charged ions (counterions) near the membrane surface and near the channel entrance, thus increasing the entry rate of counterions in the channel.

We have studied lipid bilayer surface charge effects on gramicidin channel conductance using two approaches: titration of the lipid charge by varying bulk solution pH, and dilution of a charged lipid by added neutral lipid. In the first series of experiments, we have titrated pure PS bilayers from neutral pH 7.5 down to pH 0.5, while keeping CsCl bulk concentration constant. We show that surface charge titration is masked by two factors: 1) increased contribution of protons to channel conductance when bulk solution pH goes below pH 3, and 2) channel conductance saturation with Cs^+ at neutral pH. We have also taken into account the binding of counterions (driven by their accumulation near the membrane surface) to the surface groups and the corresponding decrease in the density of charged groups on the lipid surface. By fitting the channel conductance to models based on Gouy-Chapman theory, we estimate the intrinsic pK_a^{in} of the PS headgroups.

In the second series of experiments, we have used bilayers of different PS/PC ratios at constant pH. Conductance of the gramicidin channel decreases as the charged PS is diluted by neutral PC, in qualitative agreement with our

models. Quantitatively, though, this decrease is significantly more pronounced than predicted. After checking bilayer lipid composition with the nonactin method (McLaughlin et al., 1970), we recognize that the conductance of channels in lipid mixtures is affected by additional lipid-specific factors (Providence et al., 1995; Killian, 1992; Girshman et al., 1997).

We have used a smeared-charge approach (McLaughlin, 1977) to evaluate proton and cation contributions to the gramicidin channel conductance. A numerical solution suggests that this approach is valid for a mole fraction of charged lipid in the membrane of >25% (Peitzsch et al., 1995). To determine electric potential at the charged membrane surface we considered equilibrium between counterion binding and proton titration of the lipid headgroups. Then, to estimate cation and proton concentration at the channel entrance, we use three models: 1) analytical solution for the planar Poisson-Boltzmann equation; 2) a linearized Poisson-Boltzmann equation solution; and 3) construction of a Gibbs dividing surface between the solvent bath and the charged membrane.

Despite widely noted and exhaustively analyzed concerns, electrostatic double-layer models enjoy impressive success. The expectation is that the various formulations and computations capture the space and time averages that are seen in macroscopic measurements. Nevertheless, the use of electrostatic double-layer potentials near charged macromolecules or near charged planar surfaces rests on a collection of pious hopes and miraculously compensating approximations. Electric fields vary much too rapidly to imagine that the solvent is a continuum dielectric medium with negligible structural features. Electric potentials are far too strong to say that the electrostatic energy of a mobile ion is not much different from thermal energy kT. The structure of a membrane or a macromolecule is far too detailed and varies far too widely to let us think of mathematically ideal structures.

The ionic conductance of a gramicidin ion channel in charged bilayers is a pertinent example. The channel carries so few ions per second that the solution near the channel
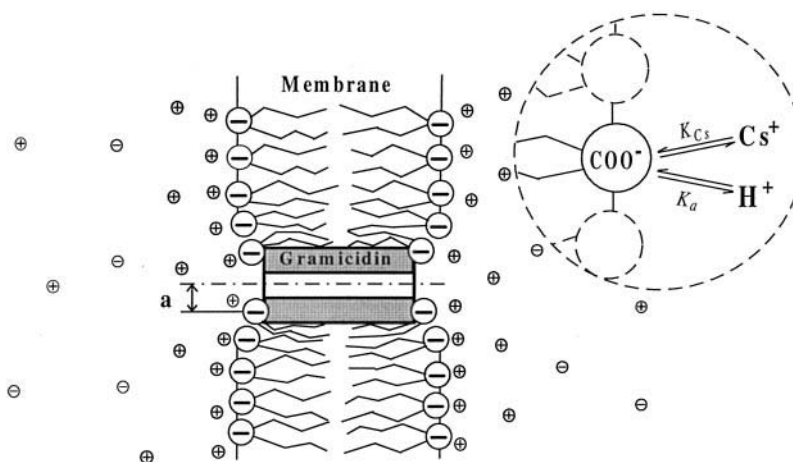

FIGURE 1   Schematic illustration of counterion accumulation near a negatively charged lipid bilayer. The gramicidin channel is shown as an uncharged cylinder. The inset shows the two charge-compensating reactions considered here: titration by protons and Cs^+ binding.

entrance should be treated as a spatially and temporally averaged region. We use this argument to introduce a spatially homogeneous equilibrium reservoir of ions (model 3)—its thickness is taken here to be the distance from the membrane to a Gibbs dividing surface, its breadth the length of diffusion of ions during the intervals between entries of successive ions (see Appendix).

MATERIALS AND METHODS

Membranes were formed by apposition of two phospholipid monolayers spread on aqueous salt solutions (Montal and Mueller, 1972). The monolayers were prepared from diphytanoyl phosphatidylcholine (PC) and diphytanoyl phosphatidylserine (PS) (Avanti Polar Lipids, Alabaster, AL). The Teflon chamber, with two compartments of 2 ml, was divided by a 15-μm-thick Teflon partition (C-Film; CHEMFAB, Merrimack, NH) with a 60–70-μm diameter aperture created by proximity to the tip of a hot platinum needle. The mixtures of PC and PS were made from aliquots of two lipids in chloroform, followed by drying lipid mixtures with nitrogen and then redissolving them in hexane to a total lipid concentration of 10 mg/ml. After bilayer formation gramicidin A (a generous gift from O. S. Andersen, Cornell University Medical College) was added from 1–10 nM ethanol stock solutions to both aqueous compartments at the amount sufficient to give a single channel activity. The aqueous solutions always contained 1 mM EDTA. For neutral pH solutions, as in the experiments with varied CsCl concentration, we used 5 mM HEPES. In experiments with PS bilayer titration, the pH of the bathing solutions was reduced by adding HCl to the chamber compartments (magnetic stirring was switched on for 2 min) followed by pH measurement in both compartments by micro pH-electrode (Orion Research, Beverly, MA). In this way we were able to form a stable membrane at low pH where PS lipids tend to form the H$_{II}$ phase (Bezrukov et al., 1995); conductance results were reversible with decreases and increases in pH. All measurements were made at continuously monitored room temperature, $T = (23 \pm 0.5)°C$.

The membrane potential was clamped using laboratory-made Ag/AgCl electrodes in 3 M KCl, 1.5% agarose bridges assembled in standard pipette tips (Bezrukov and Vodyanoy, 1993). Unless otherwise stated, membrane voltage was 100 mV. The current was amplified by a Dagan 3900 integrating patch-clamp amplifier (Minneapolis, MN) in a mixed RC mode with a 3902 headstage, recorded on a chart recorder and moved into computer memory with 16-bit resolution at a 50,000-Hz sampling rate.

The negative surface charge density of bilayers formed from a lipid mixture was estimated by the nonactin carrier conductance method of McLaughlin et al. (1970). The surface potential difference, $\Delta\psi_0$, was obtained from the cationic conductance of the nonactin-K$^+$ complex measured before (G^*) and after (G_0) the addition of impermeant electrolyte LiCl to both bulk solutions:

$$\Delta\psi_0 = -(kT/e)\ln(G^*/G_0) \qquad (1)$$

where k, T, and e have their usual meanings of Boltzmann constant, absolute temperature, and unit charge, so that $kT/e = 25.5$ mV, and $\Delta\psi_0 = \psi_0^{max} - \psi_0$. ψ_0^{max} is the maximum surface potential in the absence of Li$^+$, and ψ_0 is the surface potential at a given Li$^+$ concentration.

Bilayers from lipid mixtures were formed in the presence of 0.01 M KCl, and 6.25×10^{-7} M nonactin (Sigma, St. Louis, MO) was added with stirring in both compartments until a stable conductance was reached. LiCl in the concentration range of 10^{-3}–1 M was then added to both sides, and conductance measurements were taken after each addition. The maximum surface charge density, σ^{max}, for each lipid mixture was obtained using the combination of the Gouy equation, the Boltzmann relation, and Langmuir adsorption isotherm (Eisenberg et al., 1979; McLaughlin et al., 1981):

$$\sinh(\psi_0 e/2kT) = \frac{A\sigma^{max}}{([K^+]_b + [Li^+]_b)^{1/2}[1 + (K_K[K^+]_b + K_{Li}[Li^+]_b)\exp(-\psi_0 e/kT)]} \qquad (2)$$

where $[K^+]_b$ and $[Li^+]_b$ are the bulk K$^+$ and Li$^+$ concentrations, and K_k and K_{Li} are the association constants of K$^+$ and Li$^+$, taken to be 0.15 and 0.8 M^{-1}, correspondingly (Eisenberg et al., 1979). $A = 1/(8\ \epsilon\epsilon_0 kT)^{1/2}$, where ϵ is the dielectric constant and ϵ_0 is the permittivity of free space.

For 100% PS bilayers at pH 7.5, the nonactin conductance method yielded a surface charge density estimate of one electronic charge per 47 Å^2 (Table 1). This area agrees with data of McLaughlin et al. (1971) and Coronado and Affolter (1986), but it is less than the area of pure melted-chain phospholipids in multilayers observed by x-ray diffraction (Rand and Parsegian, 1989). The difference between electrical and structural estimates is probably due to systematic errors inherent in the nonactin method; these errors are probably less serious when one is estimating ratios of charged versus uncharged lipids in a bilayer. The PS/(PC + PS) (w/w) ratios in the planar bilayers obtained by this approach are summarized in Table 1.

TABLE 1 Calibration of surface charge density in lipid bilayers by the nonactin method

PS/(PC + PS) ratio in the lipid mixture	Surface charge density (charges/Å^2)	PS/(PC + PS) ratio in the lipid bilayer
1	1/(46.8 ± 3.3)	1
0.75	1/(77.5 ± 4.5)	0.61 ± 0.06
0.5	1/105	0.44 ± 0.03
0.25	1/204	0.23 ± 0.02

RESULTS

Fig. 2 demonstrates typical current traces measured at two different pHs for gramicidin channels in the same PS bilayer membrane. At low pH, single-channel lifetime shows a ~10-fold decrease in accordance with recent data (Lundbaek et al., 1997). What is more, the amplitude analysis shows that at pH 7.4 channel conductance is higher than at pH 3.5.

The titration of channel conductance in pure PS bilayers is shown in Fig. 3. Here channel conductance was measured in 0.1 M CsCl solutions with pH from 8.2 to 0.5. Below pH 5, conductance decreases with decreasing pH to a minimum around pH 2.5. As pH is changed to still lower values, conductance increases again and eventually even exceeds its value at neutral pH. Error bars in Fig. 3 and elsewhere represent the standard deviations; for some points they are too small to be seen.

Because the current through the channel contains contributions from both protons and Cs$^+$ ions, we measured it as a function of varied CsCl concentrations at different pHs. Table 2 shows increasing conductance as function of increasing ion and proton concentrations for channels in *neutral* PC lipids. It presents the mean conductance and its standard error of at least 50 single channel events measured for at least three different membranes in freshly prepared salt solutions. Gramicidin A channel at moderately low CsCl concentration (0.1 M) shows additivity of Cs$^+$ and H$^+$ conductance. However, at high CsCl concentration (1 M) the channel exhibits "competition" between protons and Cs$^+$ ions (for Na$^+$ ions as blockers of H$^+$ currents, see Heinemann and Sigworth, 1989).
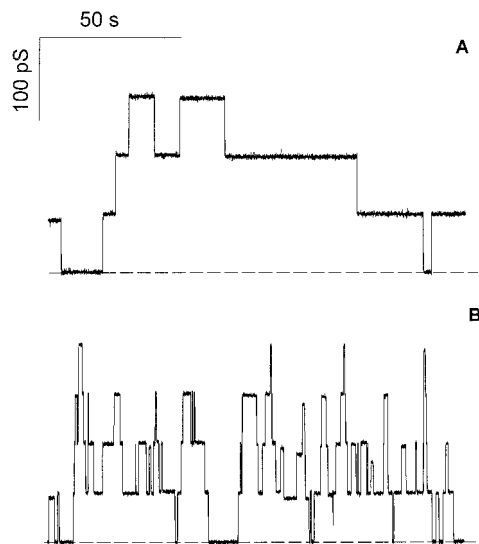
FIGURE 2   Single-channel traces of gramicidin A in PS bilayer at pH 7.4 (*A*) and 3.5 (*B*). Conditions: 0.1 M CsCl, 1 mM EDTA, 5 mM HEPES, +100 mV, 23 ± 0.5°C. The zero-current level is identified by a dashed line. Single-channel conductances are 70 pS (*A*) and 58 pS (*B*). Current records were filtered using an averaging time of 30 ms.

TABLE 2   Gramicidin channel conductance (pS) in neutral DPhPC bilayers

pH	$[Cs^+]_b = 0$	0.1 M Cs^+	0.3 M Cs^+	1.0 M Cs^+
7.0	0.0*	17.8 ± 0.9	30.2 ± 0.6	49.7 ± 3.6
3.0	5.6 ± 0.3	23.9 ± 0.5	38.4 ± 1.0	55.9 ± 2.0
2.0	42.0 ± 1.0	63.3 ± 0.7	69.0 ± 1.1	71.5 ± 1.0
1.0	191.3 ± 5.0	210.7 ± 4.6	227.8 ± 8.4	147.4 ± 7.3

* Less than 0.1 pS above the bilayer leakage conductance.

and those by Apell et al. can be attributed to the difference in physical properties of membranes formed by brushing and monolayer-apposition techniques (Montal and Mueller, 1972). Conductance measured with neutral PC saturates at ~55 pS. Conductance measured with charged PS varies little; over the 0.01–3.0 M range of CsCl concentrations it is always higher than 55 pS.

Fig. 5 shows experimental values obtained for channel conductance for different charged/neutral lipid ratios. Note that the total range of the change in conductance in these lipid mixture experiments (18 pS for pure PC to 72 pS in pure PS) exceeds the total range of conductance change in experiments with neutral lipid membranes. Compare with Fig. 4 (*bottom curve* for PC) showing that for CsCl concentrations from 0.1 M to 3 M, channel conductance changes from 18 pS to 55 pS.

Current versus voltage curves for gramicidin channels in neutral and charged lipid membranes are shown in Fig. 6. These relations are linear up to 200 mV of applied voltage for both Cs^+ ion transport (0.1 or 1 M CsCl) and proton

Fig. 4 shows the conductances of channels in neutral and charged bilayers as a function of CsCl concentration. All solutions were buffered at constant pH 7.4. The data essentially agree with previously reported values (Apell et al., 1979). The difference in absolute values between our results
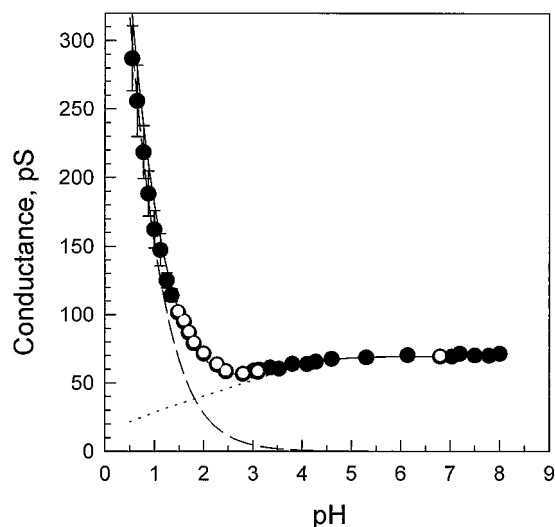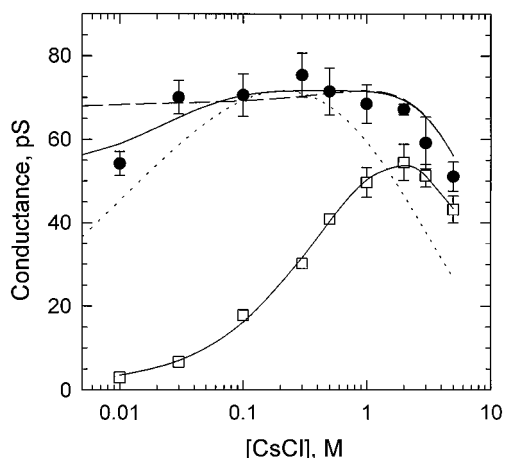

FIGURE 3   pH dependence of gramicidin channel conductance in PS bilayers. The open and closed symbols refer to different sets of measurements under the same solution conditions (0.1 M CsCl, 1 mM EDTA, 5 mM HEPES). Titration began at pH 6.8 or pH 8.0. Dashed and dotted lines show estimated contributions of protons and cesium ions, correspondingly, to total conductance *(solid line)* using procedure 3 (see also Fig. 7 *C*). Proton conductance dominates at low pH. The contribution of Cs^+ to conductance decreases monotonically with decreasing pH; neutralization of surface charge by proton binding abolishes the high-concentration reservoir of Cs^+ ions at the membrane surface drawn to the negatively charged lipid.

FIGURE 4   Gramicidin channel conductance in charged PS *(solid circles)* and neutral PC *(open squares)* bilayers as a function of CsCl concentration in the bath. Channel conductance in PC lipid bilayer is linear in salt concentration until it reaches saturation at ~1 M CsCl. Negatively charged PS lipid headgroups attract Cs^+ near the surface and the channel mouth to a concentration that saturates the channel's ion-carrying capacity. The dashed line shows the fit to PS data using a nonlinear Poisson-Boltzmann model (procedure 1) for the ion distribution; the solid line designates the fit using a linearized model (procedure 2); the dotted line represents the Gibbs dividing surface approach. The solid line through the open-square PC points is a mathematical least-squares fit with no physical model (see Discussion).
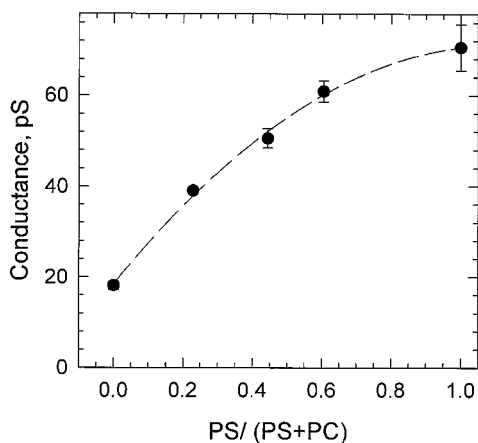
FIGURE 5   Gramicidin channel conductance in mixed bilayers as a function of PS/(PC + PS) ratio at pH 7.4 in 0.1 M CsCl. Practically all PS lipid headgroups are charged at this pH, so the surface charge is diluted in the same proportion as the content of PS in the membrane. Relative lipid composition is measured by the nonactin method (Table I). The dashed line is a second-order regression through experimental points.

transport in the presence of Cs^+ ions. It is essential to establish this ohmic linearity to know that the cationic population near the mouth of the channel acts as an inexhaustible reservoir for ion flux through the channel.

DISCUSSION

At first glance, membrane charge, pH, and CsCl concentration regulate gramicidin channel conductance in comfortably intuitive ways. Because the negative bilayer accumu-
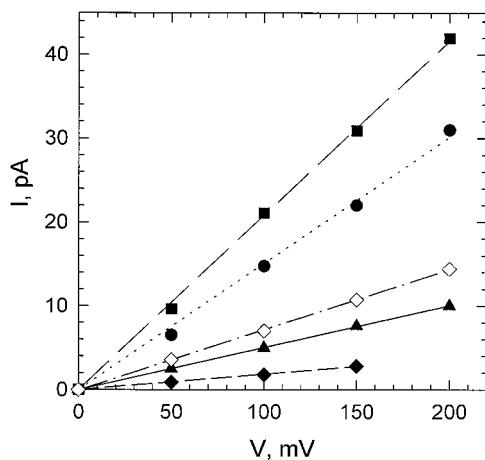

FIGURE 6   Channel conductance is ohmic. This linearity of current versus applied voltage shows that there is no depletion of ions, even with the high currents seen at 200 mV transmembrane applied voltage, both in PC *(solid symbols)* and PS *(open symbols)* bilayers. Squares, 0.1 M CsCl, pH 1.0; circles, 1.0 M CsCl, pH 1.0; triangles, 1.0 M CsCl, pH 7.4; diamonds, 0.1 M CsCl, pH 7.4. Lines are linear regressions through the data. Because of the channel's limited current-carrying capacity, the region near the mouth of the channel acts as a reliable reservoir maintained by the electrostatic double layer near the charged lipid and by rapid diffusion of ions (see Discussion).

lates positive current-carrying ions, electric currents through gramicidin are always greater in negatively charged bilayers than when gramicidin is in a neutral lipid. It is only when the geometry of the channel is considered, when the magnitude of electrostatic surface potential is computed, and when the strength of electric current is measured that we are compelled to think more carefully about the meaning of charge accumulation and the nature of the local reservoir created by charged lipid.

The issues encountered become clear with a reexamination of Fig. 1, a sketch of the peptide gramicidin incorporated into lipid. We can speak easily of an electrostatic double layer emanating from the planar membrane surface. We can speak of a distribution of ions according to that potential. We cannot speak with any assurance about the concentration of these ions in the disturbed region occupied by the peptide, especially about the mean density of ions at the channel entrance. There is no analytical solution of the double-layer equations for this difficult nonplanar geometry. Only numerical solutions are possible, and these are in progress (Murray and McLaughlin, personal communication). The operative concentration of mobile ions is nontrivial to define when one recognizes the thermal motion of the channel as well as the disorder of thermally agitated lipids.

Diffusion and disorder come into clearer view when we realize that a single-channel current of 16 pA corresponds to the passage of 10^8 charges/s. That is, there is, on average, 10 ns between entries of charge-carrying ions into the channel. Typical currents are smaller and correspond to even longer times. A peptide or lipid with a lateral mobility of 10^{-7} cm^2/s (Marsh, 1990) can move ~3 Å in the time between ion entries. Ions with solution mobilities of 10^{-5} cm^2/s will diffuse ~30 Å in this same time. Thermal motion of ions in a 30-Å region around the channel mouth equilibrates ion concentration between ion entries. If this region contains a large number of ions, we can use equilibrium distribution considerations for ionic concentrations at the channel mouth. Any careful consideration of the entry region as a reservoir or source of conducting charge must also take into account averaging produced by channel and lipid thermal motion.

We will use the nonlinear Poisson-Boltzmann approach to obtain the surface potential from a planar charged surface. From this potential we estimate the mean ion concentration at the mouth of the channel by *three different procedures*:

1. We use the Gouy-Chapman potential $\psi_{GC}(a)$ at a distance a from the surface, where a is treated as a fitted parameter, as the potential at the channel mouth (Fig. 1).
2. We multiply the surface potential $\psi_0 = \psi_{GC}(0)$ by a screening factor $\exp(-ka)$, where a is again a fitted parameter and κ is the inverse Debye screening length.
3. We locate the Gibbs dividing surface that goes with the solution of the nonlinear Poisson Boltzmann equation without further fitting; we take the counterion concen-

tration that this construction gives for the region between the membrane and the Gibbs dividing surface (see Appendix).

It will turn out that procedure 3, which explicitly recognizes the averaged nature of the counterion cloud and requires no fitting of an extra parameter (*a*, Fig. 1), gives an excellent description of change in conductance with pH. It also makes us aware of the power of the Gibbs dividing surface concept in defining an extended interface.

Titration of surface charge

We use the familiar Gouy-Chapman equation for the potential near the charged planar surface. Charge regulation comes from the dissociation and binding of Cs^+ ions (Eisenberg et al., 1979) as well as protons. Only a few attempts have previously been made to measure the intrinsic pK_a^{in} of lipid headgroups and, in particular, of the carboxyl group in PS. Most studies report apparent pK_a^{app} values, thus avoiding the complex interplay of a number of superimposed phenomena occurring at the lipid surface (interaction between adjacent ionized groups, counterion binding, change of dielectric constant, etc.). We will follow here the approach of Ninham and Parsegian (1971) to relate intrinsic pK_a^{in} to the surface charge, counterion concentration, and, thus to channel conductance. We will also point out some refinements of the theory that can presumably lead to better pK_a^{in} estimates.

We describe the titration of the PS carboxyl group by the dissociation of COOH into COO^- and H^+; we assume that Cs^+ ions bind only to the carboxyl groups in state COO^- to form neutral COOCs sites. Corresponding equilibria can be characterized, respectively, by the dissociation constants, K_a and K_{Cs}, given by

$$K_a = \frac{(COO^-)[H^+]_0}{(COOH)} \qquad (3)$$

$$K_{Cs} = \frac{(COO^-)[Cs^+]_0}{(COOCs)} \qquad (4)$$

Parentheses denote two-dimensional surface concentrations, and square brackets with subscript zero denote three-dimensional volumetric concentrations at the interface.

The lipid charge surface density σ is proportional to the surface concentration of ionized carboxyl groups $\sigma = -e \, (COO^-)$; the maximum charge density σ_0 is the density of PS lipid headgroups

$$\sigma_0 = -e[(COO^-) + (COOH) + (COOCs)]. \qquad (5)$$

From Eqs. 3–5 we obtain

$$\sigma = \sigma_0(1 + 10^{pK_a^{in}-pH_0} + [Cs^+]_0 10^{pK_{Cs}})^{-1}. \qquad (6)$$

According to the usual definitions, $pK_a^{in} = -\log K_a$, interfacial $pH_0 = -\log[H^+]_0$, and $pK_{Cs} = -\log K_{Cs}$.

All quantities in Eq. 6 refer to the lipid-solution interface and cannot be measured directly. Several models can be used to relate bulk and interfacial concentration. Considering only electrostatic effects, we have

$$[H^+]_0 = [H^+]_b \exp(-e\psi_0/kT) \qquad (7)$$

and a similar expression for bulk and surface Cs^+ concentrations. Equation 6 can now be rewritten as

$$\sigma = \sigma_0[1 + (10^{pK_a^{in}-pH_b} + [Cs^+]_b 10^{pK_{Cs}})\exp(-e\psi_0/kT)]^{-1}. \qquad (8)$$

ψ_0 is given by the Gouy-Chapman expression

$$\psi_0 = \frac{2kT}{e} \sinh^{-1}[\sigma/\sqrt{8kT\epsilon\epsilon_0([Cs^+]_b + 10^{-pH_b})}] \qquad (9)$$

In this way we obtain an implicit equation for σ that must be solved numerically for each pair of $[Cs^+]_b$ and pH_b values at a given σ_0. For a PS/PC mixed bilayer, σ_0 is reduced in the same proportion as the PS content of the mixture, PS/(PC + PS).

Titration of channel conductance

For simplicity, we assume that contributions of Cs^+ and H^+ ions to channel conductance are additive, that is,

$$G = \alpha G_{PC}([Cs^+]_{ch}) + \beta[H^+]_{ch}. \qquad (10)$$

$[Cs^+]_{ch}$ and $[H^+]_{ch}$ are cation and proton concentrations at the channel mouth, $G_{PC}([Cs^+]_b)$ is the empirical relation obtained from experiments with neutral PC lipid, α is a measured parameter accounting for the difference in channel conductance introduced by the lipid structural effect (see next section), and β is a fitted linear coefficient for the contribution from proton conductance.

Now, for Cs^+ and H^+ concentrations at the channel mouth, $[Cs^+]_{ch}$ and $[H^+]_{ch}$, we use the three procedures described above. In the first two we estimate the potential at the channel mouth, ψ_{ch}; in the last we use the average ion concentration prescribed by the Gibbs dividing surface approach.

1) $$\psi_{ch} = \frac{4kT}{e} \tanh^{-1}\left[\tanh\left(\frac{e\psi_0}{kT}\right)\exp(-\kappa a)\right] \qquad (11)$$

versus

2) $$\psi_{ch} = \psi_0 \exp(-\kappa a) \qquad (12)$$

where

$$\kappa = \sqrt{2e^2([Cs^+]_b + [H^+]_b)/kT\epsilon\epsilon_0} = 1/\lambda_D \qquad (13)$$

and thus

$$[Cs^+]_{ch} = [Cs^+]_b \exp\left(-\frac{e\psi_{ch}}{kT}\right),$$

$$[H^+]_{ch} = [H^+]_b \exp\left(-\frac{e\psi_{ch}}{kT}\right) \tag{14}$$

3) The total mobile positive-ion density is $[Cs^+]_{ch} + [H^+]_{ch} = \sigma/e\lambda_G$, where λ_G is the distance from the membrane to the Gibbs dividing surface (see Appendix), so that

$$[Cs^+]_{ch} = \frac{\sigma}{e\lambda_G} \frac{[Cs^+]_b}{[Cs^+]_b + [H^+]_b},$$

$$[H^+]_{ch} = \frac{\sigma}{e\lambda_G} \frac{[H^+]_b}{[Cs^+]_b + [H^+]_b} \tag{15}$$

since $[Cs^+]_{ch}/[H^+]_{ch} = [Cs^+]_b/[H^+]_b$.

Data analysis

The summation-of-conductance assumption, Eq. (10), is justified by the experiments with pure PC bilayers summarized in Table 2. Only at high CsCl concentrations (≥ 1 M) does the channel exhibit competition between protons and Cs^+ ions. For the "titration" of gramicidin conductance in pure PS bilayers, the effects of competition can be disregarded. For small pH values (pH < 2.5) the concentration $[Cs^+]_{ch}$ at the channel mouth does not go above 0.3 M, where Table 2 shows that competition is small; for higher pH values, concentration of $[Cs^+]_{ch}$ can increase up to 2.5 M, but the contribution of proton conductance is rapidly decreasing and is small in comparison with Cs^+ conductance.

To fit the experimental conductance titration results with theoretical curves, we relate channel conductance with $[Cs^+]_{ch}$ by using the empirical function $G_{PC}([Cs^+]_b)$ obtained from gramicidin channels incorporated in neutral lipid (Fig. 4, *bottom curve*), but we correct $G_{PC}([Cs^+]_b)$ by a factor 1.33 (α in Eq. 10) to make neutral and charged-lipid data coincide for >2 M salt concentrations. This is to account for structural factors that are inexplicable by electrostatics; different neutral-lipid reconstitutions give gramicidin conductances that differ up to a factor of 2 (Fonseca et al., 1992; for a review see Killian, 1992). The drop-off in channel conductance at high salt concentrations has been seen before (Urban et al., 1980; Apell et al., 1979; Neher et al., 1978) and ascribed to multiion interference. Because the Debye length λ_D is ≤ 3 Å for ≥ 1 M salt concentration, the laterally smoothed Gouy-Chapman equation cannot be used at these high salt concentrations when important distances such as polar group separation and a (Fig. 1) are ~10 Å. Results measured at ≥ 1 M CsCl are beyond the scope of our analysis.

The main results of the conductance pH titration for channels in pure PS bilayers bathed in 0.1 M CsCl solutions are shown in Fig. 3. In particular, we are interested in the

titration range between pH 1.5 and 6, where conductance shows a minimum near pH 2.5.

For bulk concentrations that are much smaller than the saturation concentrations (i.e., 0.1 M), the ratio between H^+ and Cs^+ conductance is close to 10. Similar values (~9) can be derived from the data by Neher et al. (1978) for 0.01 M solutions, and those by Urban et al. (1980) for 0.1 M solutions. We find that the proton conductance is also lipid dependent, which is in accordance with data of Cukierman et al. (1997). Not only do the absolute values of conductance differ (compare pH 1.0 point in Fig. 3 with corresponding data in Table 2), but, as our analysis shows, the half-saturation HCl concentration in PC (~0.07 M) is three times smaller than that in PS (~0.2 M).

Fig. 7 shows the results of fitting the data of Fig. 3 to the best pK_a^{in} using procedures 1, 2, and 3. In the first two cases it is necessary to assume an additional parameter, the effective distance a (cf. Fig. 1) between the center of the channel and the region of charged lipid. Visually reasonable fits, but with regions of systematic deviation, can be obtained by
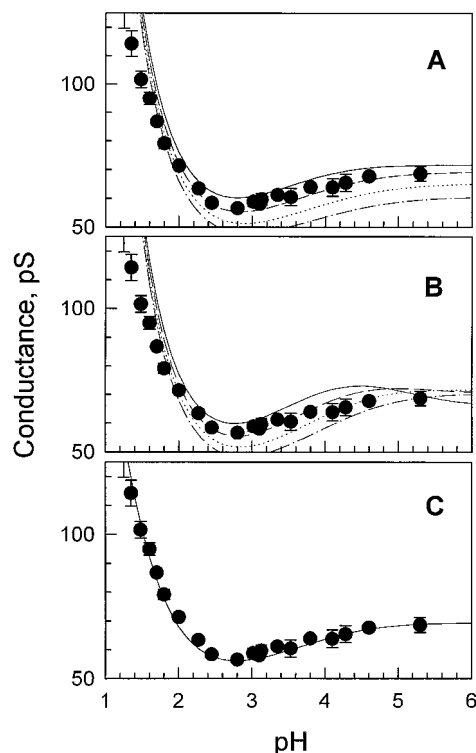
FIGURE 7 The change in conductance versus pH between pH 1.5 and 6 analyzed by three procedures to estimate ion concentration at the mouth of the channel. (*A*) Using Gouy-Chapman potential $\psi_{GC}(a)$ computed at distance a from the surface (Eq. 11). (*B*) From potential $\psi = \psi_{GC}(0)e^{-\kappa a}$ that is potential at charged surface diminished by factor $\exp(-\kappa a)$ (Eq. 12). (*C*) From Gibbs dividing surface construction (Eq. 15). In the first two procedures it is necessary to find a "best" distance a (Fig. 1) from charge lipid surface to the center of the channel. Theoretical curves for $a = 3$ Å *(solid line)*, 4 Å *(dashed line)*, 5 Å *(dotted line)*, and 6 Å *(dotted-dashed line)* are shown in the first two panels. Note sensitivity to small changes in a. For the Gibbs dividing surface procedure (*C*), no adjustable parameter is needed (except for the pK_a^{in} fitted for all three procedures).

adjusting distance a with a "best" a of 4 Å for procedures 1 and 2. Formally, a is supposed to be the sum of the channel radius (6–13 Å; Woolf and Roux, 1996) and the lipid molecule radius (4.5 Å; e.g., Parsegian and Rand, 1995), that is, we expect a value of $a \cong 10.5$–17.5 Å. However, the question is not that simple. Recent molecular dynamics study of the gramicidin channel in a lipid bilayer (Woolf and Roux, 1996), as well as old experimental observations of a significant influence of neutral lipid species on the channel conductance (for a review see Killian, 1992), suggest that the distance between the channel axis and lipid headgroups can be much smaller. The fits are very sensitive to a, and even a change of ± 1 Å in assumed a qualitatively changes theoretical predictions.

The Gibbs construction, procedure 3, with no extra parameter a, provides a relation that reproduces the main features of the data. The best-fit value of pK_a^{in} obtained here is 2.5 (compared to 2.9 and 3.0 for procedures 1 and 2). For this procedure we use Eq. 10 with Eq. 15 to compute the underlying contributions of Cs^+ conductance (dotted line, Fig. 3) and H^+ conductance (dashed line, Fig. 3). It is seen that Cs^+ conductance titration is masked by increasing proton conductance as pH goes down. However, even with the proton contribution, the channel conductance drops by ~20% from its value at neutral pH (from 72 down to 55 pS at pH 2.5). The dotted-line asymptotic limit at low pH shows calculated Cs^+ conductance for a fully titrated PS lipid.

Expectedly, the Gibbs construction loses validity when the predicted thickness of the Gibbs layer is less than the distances between charged phospholipids. Deviant behavior is seen for data collected in solutions of ≥ 0.5 M salt concentration (Fig. 4). At this concentration with a Debye length $\lambda_D = 4.3$ Å, the surface is located at a distance $\lambda_G = 7.3$ Å from the charge plane (at charge density 0.25 C/m^2 with counterion adsorption neglected), whereas the distance between charges is ~8 Å.

Although consistent with the main experimental observations and qualitatively different, all three procedures suffer from obvious limitations. First, the Poisson-Boltzmann equation is solved for cationic concentration at the ideal mathematical plane of charge, whereas the distances that we are interested in are comparable to the ~8-Å separation between charges on the surface. Second, there is no reason to believe that the potential, computed by procedures 1 and 2 as a potential distance a away from the charged lipid surface, is equivalent to a potential at the channel mouth, that is, at the center of a circle of radius a cut out of the charged plane. Third, the Gibbs procedure 3 treats proton concentration at the surface differently from the way it treats charges at the mouth of the channel. While using space-averaged ion concentration for the channel mouth, we rely on a solution of the Poisson-Boltzmann equation to calculate cesium and proton concentration at the charged surface.

Nevertheless, the pK_a^{in} estimates of 2.9, 3.0, and 2.5 found here via procedures 1, 2, and 3 differ less among themselves than the range of measurements by other methods (see review by Tocanne and Teissié, 1990).

Lipid mixtures

Fig. 5 shows experimental values for the channel conductance obtained from PS/PC bilayers containing different lipid ratios. Note from the start that the total range of conductance change in lipid-mixture experiments (18 pS for pure PC to 72 pS for pure PS) exceeds the total range of the conductance change in PC experiments (Fig. 4, bottom curve) when the CsCl concentration is varied from 0.1 M to 3.0 M (18–55 pS). This means that any electrostatic model that appeals to Cs^+ concentrations at the channel mouth only is bound to fail to describe the conductance dependence in Fig. 5. Additional lipid-dependent structural factors have to be taken into account.

Indeed, channel conductance measured in the charged lipid, PS, is higher than channel conductance in neutral lipid, PC, over the whole range of CsCl concentrations, from 0.01 M to 5.0 M (Fig. 4). This discrepancy probably reflects the influence of lipid on the channel structure. Even for neutral lipids channel conductance is not completely defined by permeant cation concentrations, but also by the particular species of lipid used to form membranes. Recent studies show that in addition to the nature of headgroups or the number of carbons in lipid acyl chains that were known to influence ion transport through the gramicidin A channel (Killian, 1992), its conductance also depends on the degree of lipid saturation (Girshman et al., 1997). Fig. 5 demonstrates that in PC/PS bilayers the surface charge influence on channel conductance is comparable to the structural influence of lipid.

To conclude: gramicidin channel conductance is a practical tool for measuring mobile-ion concentrations near an electrically charged membrane surface. The titration of membrane surface charge is seen through pH-induced changes in channel conductance; it can be converted into an estimate of the intrinsic pK_a^{in} of surface titratable groups.

Small conductance is crucial. Obeying the principle that a quantitative detector negligibly perturbs the system it would observe, the conductance of gramicidin is negligible compared to the "conductive capacity" of the salt solutions from which it feeds and into which it voids. There is virtually no voltage drop across those salt solutions and no perturbation of the ionic gradients already set up by the charged membrane.

Kinetically speaking, the intervals between passage of ions through gramicidin are long compared to the times needed for ions to diffuse to the mouth of the channel. The volume of solution from which ions can be withdrawn, computed from diffusion lengths, contains ~20 cations on average, so that the withdrawal of one ion into the channel is a small perturbation.

The Poisson-Boltzmann equation provides the electrostatic potential near the charged membrane surface. In our

first two procedures, we calculate the local counterion concentration at the channel mouth. These calculations require an additional fitting parameter of potential screening between charged lipid and channel mouth. Our third procedure uses the electrostatic potential to determine the Gibbs dividing surface for the distribution of mobile ions. The reservoir from which the channel draws mobile charge is treated as having a concentration averaged over a region of finite thickness bounded by that surface. The description of conductance change with pH requires no additional parameters. This previously unrecognized Gibbs construction might be a practical means of expressing time and space averages over fluctuating charged surfaces.

APPENDIX: LOCATION OF THE GIBBS DIVIDING SURFACE AT A DISTANCE λ_G FROM A CHARGED MEMBRANE

We use the Gouy-Chapman equation, the exact result for the nonlinear Poisson-Boltzmann equation solved for a single charged surface immersed in a salt solution,

$$\frac{e\psi(z)}{kT} = \ln\left(\frac{1 - \alpha(z)}{1 + \alpha(z)}\right)^2 \qquad (1a)$$

where $\alpha(z) \equiv \gamma e^{-z/\lambda_D}$ and γ is the positive root of a quadratic equation

$$\gamma^2 + \frac{\epsilon\epsilon_0 kT}{\pi e|\sigma|\lambda_D}\,\gamma - 1 = 0 \qquad (2a)$$

In the limit $\sigma \to \infty$, $\gamma \to +1$, when $\sigma \to 0$, $\gamma \to \pi e|\sigma|\lambda_D/\epsilon\epsilon_0 kT \to 0$.

The Gibbs prescription is to create step functions in the ion distributions with the step occurring at a "dividing surface." In thermodynamic terms, this dividing surface *defines the boundary* between the charged lipid surface with its counterions and the undisturbed salt solution phase in contact with it. The position λ_G of this dividing surface is determined unambiguously from the constraint that the total number of ions described by the step functions is equal to the number of ions estimated from the continuous distribution that is created by $\psi(z)$.

The negative ion density distribution

$$n_-(z) = n(\infty)\exp[e\psi(z)/kT] = n(\infty)\left(\frac{1 - \alpha(z)}{1 + \alpha(z)}\right)^2 \leq n(\infty) \qquad (3a)$$

indicates a deficit $(n_-(z) - n(\infty))$ of negative mobile ions near a negatively charged surface. This deficit is integrated from $z = 0$ to ∞ and compared with the deficit of a step function whose value is zero from $z = 0$ to λ_G and $n(\infty)$ from λ_G to ∞.

The comparison can be written

$$\int_0^\infty (n_-(z) - n(\infty))\mathrm{d}z = -\lambda_G n(\infty) \qquad (4a)$$

Explicitly,

$$n(\infty)\int_0^\infty\left(\left(\frac{1 - \alpha(z)}{1 + \alpha(z)}\right)^2 - 1\right)\mathrm{d}z = -4n(\infty)\int_0^\infty \frac{\alpha(z)}{(1 + \alpha(z))^2}\,\mathrm{d}z$$
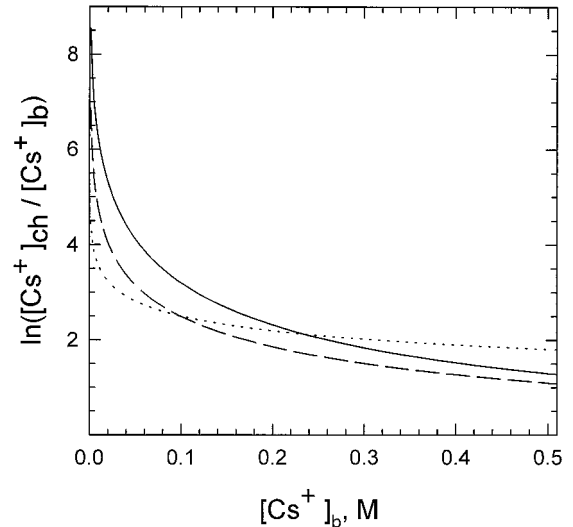
$$= -\lambda_G n(\infty) \qquad (5a)$$

FIGURE 8   The change in relative concentration of Cs^+ ions near the mouth of the channel versus bulk solution as a function of Cs^+ concentration in bulk analyzed by three procedures for pH 7.4. The dashed line shows the nonlinear Poisson-Boltzmann model (procedure 1); the solid line is computed using a linearized model (procedure 2); the dotted line represents the Gibbs dividing surface approach. In the first two procedures theoretical curves were derived for $a = 4$ Å. For all three procedures $pK_a^{in} = 3.0$.

so that

$$\lambda_G = 4\lambda_D\frac{\gamma}{1 + \gamma} \qquad (6a)$$

By the electroneutrality of the entire system, the total number of (mobile) positive ions must equal the number of negative mobile ions plus the number of negative charges fixed to the surface. For this reason, the corresponding step function for positive charge has the value n_s for $z \geq \lambda_G$ and a constant value, $\sigma/e\lambda_G$, for $0 \leq z \leq \lambda_G$. The surface at $z = \lambda_G$ divides the space near the charged surface into a region of undisturbed salt solution and a region depleted of all negative ions and average mobile positive ions at a concentration $\sigma/e\lambda_G$.

Fig. 8 compares Cs^+ concentrations at the channel mouth (plotted as ratios to Cs^+ concentration in the bulk) for the three different approaches. A "smoothing effect" of the Gibbs dividing surface construction (*dotted line*) is clear.

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