Conducting Gramicidin Channel Activity in Phospholipid Monolayers

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ABSTRACT Potential step amperometry (chronoamperometry) of the Tl(I)/Tl(Hg) electrochemical reduction process has been used to investigate the underlying mechanisms of gramicidin activity in phospholipid monolayers. The experiments were carried out at gramicidin-modified dioleoyl phosphatidylcholine (DOPC)-coated electrodes. Application of a potential step to the coated electrode system results in a current transient that can be divided into two regions. An initial exponential decay of current corresponds to the inactivation of monomer channel conductance and a longer time scale quasi-steady-state represents the diffusion of ions to a bimolecular surface reaction. Concentrations of monomer conducting channels are relatively low, and the results indicate that two or more forms of gramicidin are in equilibrium with each other in the layer. Aromatic/conjugated compounds incorporated into the monolayer increase the reduction current by decreasing the rate of channel inactivation and increasing the stability of the conducting channel. This effect is positively correlated with the degree of the compound's aromaticity. The anomalous influence of alkali metal ions on the reduction current is consistent with the model of gramicidin being speciated in the monolayer in more than one form. The results have implications on the lability of the peptide conformation in biological membranes and its dependence on lipid environment, solution composition, and applied potential.

GLOSSARY

DOPC	dioleoyl phosphatidylcholine
PS	phosphatidylserine
DOPC-0.3 PS	DOPC with 0.3 mole fraction PS
DOPC-0.12 retinol	DOPC with 0.12 mole fraction all-*trans* retinol
A	electrode area
$i(t)$	current transient
i	current sampled at specified time of current transient
E	potential applied to monolayer-coated electrode
t	time
C_rE_r	electrode mechanism of a homogeneous chemical reaction preceding a rapid electron transfer
k_1^{het}	heterogeneous rate constant of passage of Tl^+ across gramicidin-modified monolayer
D	diffusion constant of Tl^+ ion in bulk solution
c_0	bulk solution concentration of Tl^+
$i_{t=0}$	quasi-steady-state current extrapolated to $t = 0$
τ	time constant of initial exponential decay of current transient
k	rate constant of initial exponential decay of current transient
k_1	first-order forward rate constant of hypothetical homogeneous chemical reaction preceding electron transfer
k_{-1}	first-order backward rate constant of hypothetical homogeneous chemical reaction preceding electron transfer
$k = k_1 + k_{-1}$	
$K = k_1/k_{-1}$ and $B = K^2k/(1 - K^2)$	
l	length of gramicidin channel in monolayer and thickness of monolayer
k_1'	first-order rate constant describing the transport of Tl^+ across the gramicidin-modified monolayer
k_{in}'	second-order rate constant describing Tl^+ entry into and translocation through the monomer gramicidin channel
A_v	Avagadro's number
r	radius of the gramicidin $\beta^{6.3}$ helix channel lumen in monolayer
$1 - \theta$	fractional area of electrode rendered electroactive in the gramicidin-modified monolayer
C	capacitance of gramicidin-modified monolayer-coated electrode
C_{lip}	capacitance of unmodified DOPC phospholipid monolayer-coated electrode
C_{Gr}	hypothetical capacitance of gramicidin monolayer-coated electrode
x	fractional area of electrode covered by gramicidin
Gr	nonconducting species of gramicidin in monolayer
Gr*	conducting species of gramicidin in monolayer
k_+	first-order rate constant of activation of gramicidin channels in monolayer
k_-	first-order rate constant of inactivation of gramicidin channels in monolayer
$k = k_+ + k_-$	
N	surface concentration of total gramicidin
χ_{Gr}	fraction of gramicidin in monolayer defined as if every molecule in the monolayer occupied the surface area of a conducting channel pore of radius r
$[Gr_T]$	volume concentration of total gramicidin in monolayer
[Gr]	volume concentration of nonconducting gramicidin in monolayer
[Gr*]	volume concentration of conducting gramicidin in monolayer
$[Gr^*K^+]$	volume concentration of K^+-conducting gramicidin complex in monolayer
$[Gr^*Tl^+]$	volume concentration of Tl^+-conducting gramicidin complex in monolayer
β'_{Gr^*K+}	stability constant of K^+-monomolecular gramicidin channel complex in monolayer

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β'_{Gr*Tl^+}	stability constant of Tl^+-monomolecular gramicidin channel complex in monolayer
β''_{Gr*K^+}	stability constant of K^+-bimolecular gramicidin channel complex in bilayer
β''_{Gr*Tl^+}	stability constant of Tl^+-bimolecular gramicidin channel complex in bilayer
$[K+]$	solution bulk concentration of K^+
$[Tl^+]$ $(= c_0)$	solution bulk concentration of Tl^+

INTRODUCTION

The influence of biologically active compounds on ion channel function in biological membranes is always of great interest (Franks and Lieb, 1994, 1997, 1998, 1999; Hendry et al., 1985; Elliot and Haydon, 1986; Haydon et al., 1977; Haydon and Urban, 1983a,b,c; Narahashi, 2000; Tang et al., 2000). The reasons for this are twofold. First, from a fundamental point of view it has often been proposed that biologically active compounds exert their effect at the biological membrane level by modifying ion channel function (Franks and Lieb, 1994, 1997, 1998, 1999; Narahashi, 2000; Tang et al., 2000). Thus, investigations on the way in which the compounds interact with ion channels increases the understanding of toxicity mechanisms. Second, with respect to applications in the field of sensor technology there is an interest in developing a model ion channel system that selectively responds to toxic compounds (Woodhouse et al., 1999; Raguse et al., 1998; Cornell et al., 1997). These model ion channel systems could subsequently be used as sensing elements for toxicity detection.

One membrane model that has been used to study ion channel activity is that of a phospholipid-coated mercury electrode with incorporated gramicidin (Nelson, 1991, 1993, 1996, 1997; Nelson and Bizzotto, 1999). The phospholipid coating is a monolayer where the phospholipid heads are in the solution and the phospholipid tails are next to the mercury (Miller et al., 1976, Nelson and Auffret, 1988; Leermakers and Nelson, 1990; Nelson and Leermakers, 1990), representing exactly half a bilayer. The gramicidin in channel form was shown to facilitate the transport of Tl^+ across the ion-impermeable phospholipid monolayer (Nelson and Bizzotto, 1999). It was concluded that the $\beta^{6.3}$ helix monomer of gramicidin is responsible for the conduction of Tl^+ across the monolayer. This was based on the fact that the monomer $\beta^{6.3}$ helix length of 1.3 nm (Hladky and Haydon, 1984) is equal to the thickness of the monolayer (Nelson and Auffret, 1988). There is little probability that the double stranded form of gramicidin if present facilitates Tl^+ conduction because its length of 2.6–3 nm is twice that of the monolayer thickness (Dhathathreyan et al., 1988). The orientation of the $\beta^{6.3}$ helix monomer in the monolayer is also of importance. A weight of evidence exists that shows that the clustering of gramicidin tryptophan residues at the lipid-water interface is critical to the structure of the $\beta^{6.3}$ helix in phospholipid bilayers (O'Connell et al., 1990; Koeppe et al., 1999). There is every reason to assume that the same is true in phospholipid monolayers because in principle a phospholid bilayer consists of one monolayer adsorbed back to back on another. Indeed, the gramicidin bimolecular channels are formed from two $\beta^{6.3}$ helices in each of the individual monolayers of the bilayer that come together to form a dimer (O'Connell et al., 1990; Cross et al., 1999; He et al., 1994). In some cases, 70% of the gramicidin in the monolayers of the bilayer exists as separate $\beta^{6.3}$ monomers (He et al., 1994). Previous work showed that the properties of the lipid monolayer of a bilayer and those of the lipid monolayer on mercury are very similar. In particular, the characteristics of the lipid-water interface in both systems are identical (Leermakers and Nelson, 1990). As a result of these considerations, it is assumed throughout this paper that the $\beta^{6.3}$ helix monomer with the tryptophan residues clustered at the phospholipid-water interface conducts Tl^+ across the phospholipid monolayer.

The supported gramicidin-modified phospholipid monolayer model is the simplest biological membrane model available, but its versatility and robust nature allow for very precise and accurate experiment. It has already been shown that the gramicidin channel's transporting function in this membrane system is selectively sensitive to both aromatic/conjugated compounds incorporated into the monolayer (Nelson, 1996, 1997; Nelson and Bizzotto, 1999) and also to solution composition (Nelson, 1993, 1996). One of the aims of this study, therefore, was to investigate the origin of the sensitivity of the gramicidin channel to aromatic/conjugated compounds. Another aim was to investigate whether the ion permeation mechanism was related to the structural dynamics of gramicidin, which are still a subject of controversy (Cross et al., 1999; Burkhart and Duax, 1999).

This investigation looks at the ion-transporting properties of the gramicidin channel in phospholipid monolayer environments containing increasing concentrations of aromatic hydrocarbons with increasing aromaticity, respectively. In particular, three-, four-, and five-membered polynuclear aromatic hydrocarbons, namely, phenanthrene, pyrene, and benzo-α-pyrene, respectively, and the highly conjugated all-*trans* retinol were incorporated into gramicidin-modified monolayers. The effects on the gramicidin channel activity were compared with those of different electrolytes and the negatively charged phosphatidylserine (PS) in the monolayer to gain more insight into the interaction mechanisms.

The electrochemistry of the Tl(I)/Tl(Hg) couple was studied at coated electrodes with gramicidin in solution. The Tl^+/Tl(Hg) redox process on an uncoated mercury surface is very rapid (Blankenborg et al., 1993; Galus, 1993), and thus its electrochemical kinetics on a gramicidin-modified DOPC-coated surface are limited by the passage of the ion through the channel. This study specifically looks at the Tl^+ reduction process in which after translocating though the gramicidin channel, Tl^+ is reduced to Tl(Hg) and passes into the mercury as Tl(Hg). A chronoamperometric experimental procedure was employed applying potential steps in

the potential region covering and at more negative potentials than those of the Tl^+/Tl(Hg) redox process. Previous studies had been performed in a similar way using chronoamperometry as the electrochemical technique (Nelson and Bizzotto, 1999). Although those studies provided some insight into the mechanism, a comprehensive investigation of the processes that occur at short time scales had not been done. In the present work, potential step experiments were carried out where special attention was paid to the current transient between 1 and 40 ms from the application of the pulse.

MATERIALS AND METHODS

Electrolytes were fully de-aerated with special-grade argon before each experiment, and a blanket of argon gas was maintained above the electrolyte during the experiment. Monolayers of DOPC (semi-synthetic grade, Lipid Products, UK) and mixed monolayers of DOPC and PS (grade 1, bovine spinal cord, Lipid Products, Red Hill, Surrey, UK), all-*trans* retinol (Sigma Chemicals, Poole, UK), phenanthrene, pyrene, and benzo-α-pyrene were prepared as before (e.g., Nelson et al., 1990) by mixing DOPC and the required mole fraction of the compound in pentane and spreading at the gas-water interface (area = 28 cm^2) in the electrochemical cell (volume = 50 cm^3). Such monolayers are referred to in the text as the lipid-mole fraction compound. *All-trans* retinol, phenanthrene, pyrene, and benzo-α-pyrene were initially prepared in acetone. Monolayers at the gas-water interface were modified with gramicidin D (Sigma) by adding volumes of 2.13×10^{-4} and 2.13×10^{-5} mol dm^{-3} gramicidin to the electrolyte from methanol stock solutions (e.g., Nelson and Bizzotto, 1999). Ten minutes were allowed to enable incorporation of the added gramicidin into the gas-water interface monolayer. Gramicidin D is a mixture of gramicidins A, B, and C in the approximate ratio of 72:9:19, respectively (Hladky and Haydon, 1984). A fresh mercury drop of area $A = 0.0088$ cm^2 was coated with the monolayer from the gas-water interface before each experiment. The electrolytes used were 0.1 mol dm^{-3} KCl, CsCl, NaCl, and LiCl prepared from pre-combusted salts (BDH Chemicals, Poole, Dorset, UK). For the experiments with DOPC-0.3-PS-coated electrodes, electrolytes were prepared to 0.001 mol dm^{-3} concentration with phosphate buffer (BDH) to control the pH to 7.

The experiments consisted of a series of potential steps applied to the monolayer. In these, the electrode was pulsed from -0.2 V to successively more negative voltages from -0.3 to -0.7 V at 0.025-V intervals. The duration of each pulse was 0.04 s. Between each pulse the electrode was held at -0.2 V for >10 s to remove the reduced ion from the mercury. In the pulsing experiments, the current ($i(t)$) was recorded at a 40-kHz sampling frequency with a 20-kHz low-pass pre-filter. The current transients were analyzed in two ways. First, the current values at 10 ms following the initiation of the pulse were plotted versus the potential to give sampled current versus potential (i vs. $-E$) plots. Second, the exact equation (Macdonald, 1977) describing the current transient arising from a chemical step preceding the rapid electron transfer (C_rE_r) process was also applied. In this, the current transient from 1 to 40 ms was fitted to the $i(t)$ vs. t equation. A curve-fitting routine in the IGOR analysis program (Wavemetrics, Oswego, OR) was used for this. Current data at shorter time scales than 1 ms were not included in the fit because of errors due to the charging current. This procedure was carried out for all current transients from potential steps to between -0.5 and -0.7 V with Tl^+ as the electroactive ion.

Forward and back scans of potential steps were carried out on two to three replicate coated electrode systems. Mean values of the current and the kinetic parameters derived from the forward and back scans of potential steps and thus for the replicate experiments were estimated. The ranges of results of the replicate experiments were displayed as error bars. At the end of the experiment, an AC voltammogram of the coated electrode was recorded to check the integrity of the layer. In these measurements, the cell was stimulated with an AC waveform (75 Hz, 0.0045 V rms) superimposed on a voltage ramp (scan rate, 5 mV s^{-1}). The out-of-phase and in-phase components of the current were recorded. In the experiments, the concentration of electroactive ion in solution was 10^{-4} mol dm^{-3} added from a working solution of $TlNO_3$ (Sigma). Experiments with all-*trans* retinol were performed under dim red light because of the light sensitivity of the compounds.

Measurements were carried out using a Metrohm potentiostat (E506 Polarecord), and data were recorded with a Maclab (16-bit, 100-kHz) data acquisition system. The Maclab system (A and D Instruments, Hastings, Sussex, UK) was also used to stimulate the cell potential. The Metrohm potentiostat in combination with a PAR 5110 lock-in amplifier was employed to record the AC voltammograms of the coated and uncoated electrodes. All potentials in this study are quoted versus the Ag/AgCl:3.5 mol dm^{-3} KCl reference electrode.

RESULTS

Fig. 1 *a* displays a characteristic current transient resulting from the application of a voltage step to the gramicidin-modified phospholipid-coated electrode. The form of the current transient can be divided into two regions. The longer time scale region represents a quasi-steady-state. This fits the model describing the diffusion of an ion to a surface heterogeneous reaction described by the equation (Ovchinnikov et al., 1989):

$$i = FAc_0k_1^{het}\exp((k_1^{het\,2}/D)t)\mathrm{erfc}((k_1^{het}/D^{1/2})t^{1/2}), \quad (1)$$

where k_1^{het} is the heterogeneous rate constant of the surface bimolecular reaction, D is the diffusion coefficient taken in this study as 2×10^{-5} cm^2 s^{-1} for Tl^+ (Kolthoff and Lingane, 1952), and c_0 is the bulk concentration of the ion in solution. Eq. 1 is numerically similar to the solution to the problem of the diffusion of an ion to a random array of ultramicroelectrodes (Scharifker, 1988). The value of k_1^{het} can be estimated by extrapolation of the plot to $t = 0$ where the current, $i_{t=0}$ is expressed as

$$i_{t=0} = FAc_0k_1^{het}. \quad (2)$$

Significantly, at shorter time scales, the current transient is characterized by an exponential decay or relaxation with time constant $\tau = k^{-1}$.

The entire current transient fits the model describing a homogeneous chemical reaction preceding a rapid electron transfer (C_rE_r mechanism). The equation characterizing this model is written as (Macdonald, 1977)

$$\begin{aligned} i(t) = {} & [FAD^{1/2}c_0/(1-K^2)] \\ & \times \{[K(e^{-kt}-K)/\pi^{1/2}t^{1/2}] \\ & + e^{Bt}(\{K(k+B)^{1/2} \times \mathrm{erf}[(k+B)^{1/2}t^{1/2}]\} \\ & - B^{1/2}\mathrm{erf}(B^{1/2}t^{1/2}))\} \end{aligned} \quad (3)$$

In Eq. 3, $k = k_1 + k_{-1}$, and k_1 and k_{-1} are the forward and reverse homogeneous rate constants, respectively, of the

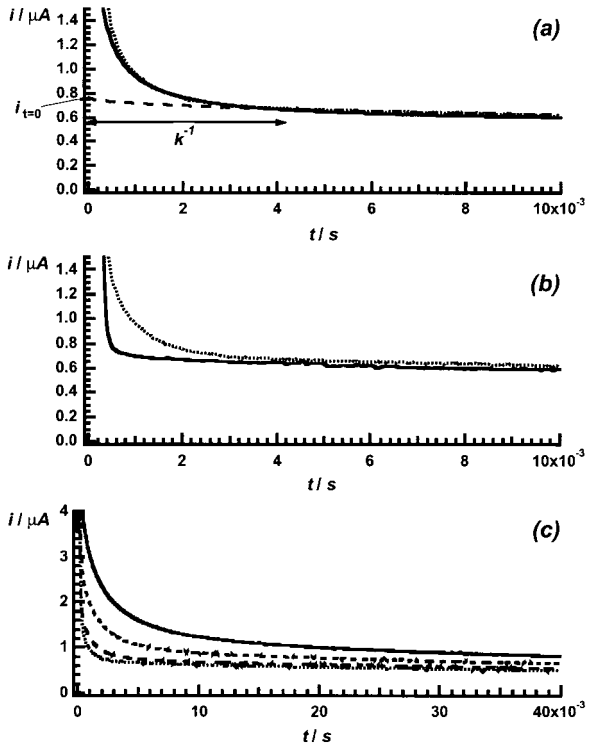

FIGURE 1 Current transients in response to potential steps applied to DOPC-coated electrodes in 0.1 mol dm^{-3} KCl with 10^{-4} mol dm^{-3} Tl^+. Transient (···) arising from potential step −0.2 to −0.55 V with 12.7 nmol dm^{-3} gramicidin in solution. (*a*) Fit of the exact (——) and approximate (– – –) equation describing the current transient resulting from a C_rE_r electrode mechanism following a potential step. (*b*) Current transient (——) arising from potential step (−0.2 to −0.8 V) applied to DOPC-coated electrode with no gramicidin in solution. At −0.8 V, defects are formed in the DOPC monolayer and it becomes permeable to ions. (*c*) Current transients in order from bottom to top, respectively, arising from potential steps (−0.2 to −0.55 V) applied to DOPC, DOPC with 2.2% (– – –), 6.2% (- - -), and 11.6% (——) all-*trans*-retinol-coated electrodes with 12.7 nmol dm^{-3} gramicidin in solution.

chemical step of which K ($= k_1/k_{-1}$) is the equilibrium constant. k represents the rate of attainment of chemical equilibrium before the charge transfer. $B = K^2k/(1 - K^2)$. The important feature of Eq. 3 is that it is valid for all values of K provided $k_{-1} > k_1$ and for current data obtained over all real time. This equation simplifies to Eq. 1 at longer time scales where $k_1^{het} = Kk^{1/2}D^{1/2}$ (Nelson and Bizzotto, 1999). Expressed in this way, Eq. 1 is referred to as the approximate equation describing the potential step current transient resulting from the C_rE_r mechanism (Galus, 1993). When Eq. 2 is fitted to the current transients, the time constant of the initial exponential decay corresponds to $\tau = k^{-1}$.

Fig. 1 *b* shows that the initial exponential decay of the current is characteristic of current transients obtained with gramicidin channels incorporated into the phospholipid. When an unmodified phospholipid monolayer is pulsed to a potential (−0.8 V) where the monolayer itself becomes permeable through defect formation (Bizzotto and Nelson, 1998), no such exponential decay of current resulting from Tl^+ reduction is observed. In the latter case, the current rapidly decays from the charging current peak to the quasi-steady-state as described by Eq. 1. The initial exponential decay and the current value of the transients resulting from the pulse applied to the gramicidin-modified lipid-coated electrodes is influenced by the lipid environment and the electrolyte composition. Fig. 1 *c* shows the effect of the progressive incorporation of all-*trans* retinol in the monolayer on the respective current transients where the current and $\tau = k^{-1}$ increase.

The influence of aromatic/conjugated compounds incorporated in the monolayer on increasing $\tau = k^{-1}$ is summarized in Fig. 2 *a*. In this figure, an increase in the aromaticity of a compound correlates with a decrease in the value of k or an increase in τ. Fig. 3 *a* shows plots of the current sampled at 10 ms of the transient versus the applied potential (i vs. $-E$ plots) from potential steps applied to gramicidin-modified DOPC-coated electrodes in different electrolytes. The effect of the electrolyte ion, Li^+, in significantly depressing the reduction current is evident. In Fig. 3 *a*, the

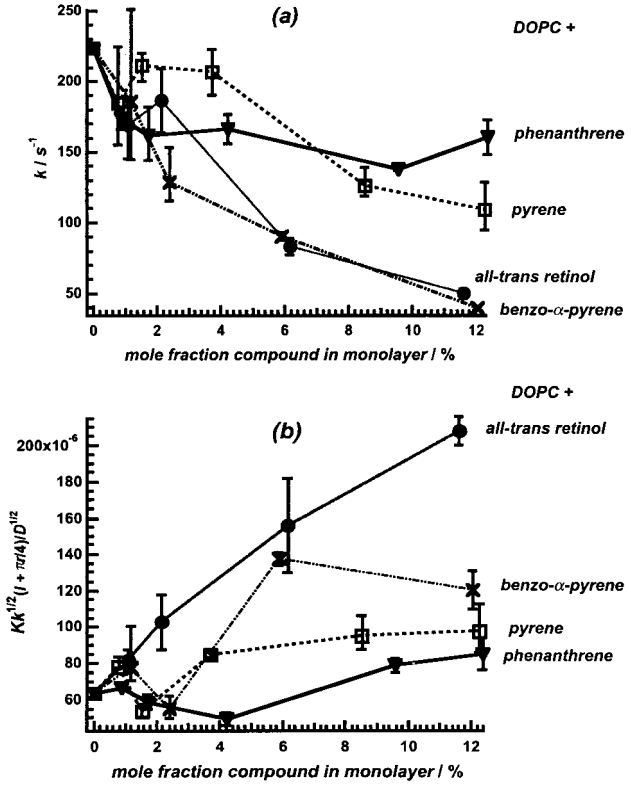

FIGURE 2 Values of k (*a*) and $Kk^{1/2}(l + \pi r/4)/D^{1/2}$ (*b*) from potential step (−0.2 to −0.55 V) experiments at DOPC with phenanthrene (▼), pyrene (□), benzo-α-pyrene (×) and all-*trans* retinol (●) coated electrodes plotted versus the percent mole fraction compound in the monolayer. Experiments carried out in 0.1 mol dm^{-3} KCl with 10^{-4} mol dm^{-3} Tl^+ and 12.7 nmol dm^{-3} gramicidin in solution.

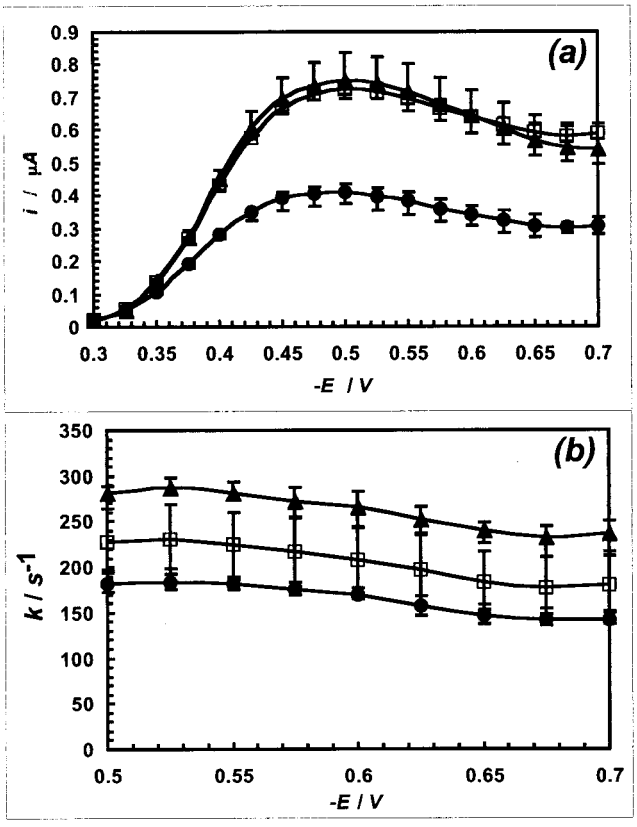

FIGURE 3 Values of i sampled at 10 ms (a) and k (b) from potential step (from −0.2 V) experiments at DOPC-coated electrodes versus the potential ($-E$) of the step. Experiments were carried out in 0.1 mol dm^{-3} LiCl (●), NaCl (□), and CsCl (▲) with 12.7 nmol dm^{-3} gramicidin in solution.

current increases between the potentials of −0.3 and −0.5 V are a function of the Tl^+/Tl(Hg) redox process and the relative stabilities of the Tl^+ ion and the Tl(Hg) species on the mercury interface. At potentials more negative than −0.5 V, the concentration of the Tl^+ ion on the mercury surface is virtually zero; thus, any changes in current are a function of the gramicidin channel conducting activity. Accordingly, a decrease in gramicidin channel conducting activity is noted between potentials −0.5 and −0.7 V.

DISCUSSION

Current transients

The surface reaction modeled by Eq. 1 involves the permeation of monomer channels by Tl^+. The value of k_1^{het} given by Eq. 2 can be divided by the thickness of the monolayer ($l = 1.3 \times 10^{-7}$ cm (Nelson and Auffret, 1988)) to give the frequency of ions crossing the monolayer, k_1', as a first-order rate constant. From the transient in Fig. 1 a, where $K = 0.13$, $k = 234.7$, and $k_1^{het} = Kk^{1/2}D^{1/2} = 8.90 \times 10^{-3}$ cm s^{-1}, $k_1' = k_1^{het}/(1.3 \times 10^{-7}\ \text{cm}) = 6.84 \times 10^4\ \text{s}^{-1}$. The entry and passage of the Tl^+ ion through the bimolecular gramicidin channel has been reported as being diffusionally controlled (Andersen and Feldberg, 1996). Even in channel systems where ion translocation is rate limiting; diffusion up to the channel opening becomes the rate-determining process at low permeant ion concentrations (Busath et al., 1998). The diffusionally controlled second-order reaction rate constant (k_{in}') for channel transport has been variously reported (e.g., Andersen and Feldberg, 1996), but the simplest estimate is given by Hille (1992). This treatment can be equally applied to monomer channels in monolayers where only diffusional control is considered and the channel profile is not significant. This approach takes account the access and transport of the ion to and through the channel, respectively, and is

$$k_{in}' = \pi r^2 D A_v/(l + \pi r/4) = 1.028 \times 10^{11}\ \text{mol}^{-1}\ \text{cm}^3\ \text{s}^{-1}, \quad (4)$$

where r is the radius of the lumen of the channel, which is 2 Å (Hladky and Haydon, 1984); l is the length of the channel, which is the same as the monolayer thickness and is 1.3×10^{-7} cm (Hladky and Haydon, 1984); and A_v is Avagadro's number. Therefore, the surface concentration of $\beta^{6.3}$ gramicidin facilitating the permeation of ions when $i_{t=0} = 0.78\ \mu$A (see Fig. 1 a) is

$$k_1'/k_{in}' = (6.85 \times 10^4\ \text{s}^{-1}/1.028 \times 10^{11}\ \text{mol}^{-1}\ \text{cm}^3\ \text{s}^{-1}) \times 10^{-3} \times 1.3 \times 10^{-7}\ \text{cm} = 8.66 \times 10^{-14}\ \text{mol cm}^{-2}. \quad (5)$$

This value is relatively small and represents 3.33×10^{-4} mol fraction of the monolayer if the phospholipid surface concentration is 2.6×10^{-10} mol cm^2 (Moncelli et al., 1994). This mole fraction of active conducting gramicidin renders a fractional electrode area ($1 - \theta$) of 6.57×10^{-5} electroactive. From $1 - \theta$ can be calculated the average distance between the pores (Amatore et al., 1983; Finklea et al., 1993) of $r/(1 - \theta)^{1/2} = 2.5 \times 10^{-6}$ cm. Thus, the time scale necessary for the preliminary overlap of the diffusion hemispheres around each pore is $(2.5 \times 10^{-6}\ \text{cm}/D^{1/2})^2$ or 0.3 μs from the beginning of the pulse. Clearly, after 1 ms, there will be considerable overlap of the diffusion hemispheres, and pure radial diffusion will not be a significant factor affecting the electrochemical results.

The origin of the exponential current decay at short time scales can be seen as an initial relaxation process. Such relaxation processes are characteristic of ion-channel-mediated current transients (Hille, 1992; Colquhoun and Hawkes, 1994). The millisecond time constant of the relaxations represents the closing or inactivation of permeant channels rather than channel transport processes. The reason for this is that relaxations associated with channel transport mechanisms have a very much shorter time scale of nanoseconds (Gates et al., 1990). The mechanism behind the monomer channel inactivations giving rise to the current

transients in Fig. 1 is different from the well characterized bimolecular gramicidin channel closing events (Bamberg and Lauger, 1973). In the case of monomer channel inactivations, the $\beta^{6.3}$ species is converting to another nonconducting peptide conformation in the monolayer. On the other hand, the slower closing and inactivation of the dimeric gramicidin channel in bilayers (Bamberg and Lauger, 1973) is caused by the two $\beta^{6.3}$ species in each monolayer of the bilayer dissociating and floating away from each other in opposite monolayers (He et al., 1994). The $\beta^{6.3}$ monomer in the monolayer on mercury will be inherently less stable than the gramicidin dimer because of its six unsatisfied hydrogen bonds oriented toward the mercury, which may account for its shorter lifetime.

Model

Three factors suggest that the concentration of total gramicidin in the layer is larger than that of the conducting gramicidin and that the level of conducting gramicidin can alter depending on the experimental conditions. This is commensurate with previous findings that have shown that gramicidin exists in a variety of conformations in lipid monolayers (Naydenova et al., 1995; Vila et al., 1996; van Mau et al., 1988; Dhathathreyan et al., 1988; Ogoshi and Mita, 1997; Ulrich and Vogel, 1999). First, gramicidin at 12.7×10^{-9} mol dm^{-3} electrolyte concentration in the absence of Tl^+ increases the capacitance of the DOPC monolayer significantly at -0.4 V by $\sim$2.5% from 1.72 to 1.76 μ F cm^{-2} (see Fig. 2 *c*) (Nelson and Bizzotto, 1999). The double-layer capacitance, C, of the lipid-covered electrode consists of a linear contribution from the capacitance of the lipid domain, C_{lip}, and that of the gramicidin, C_{Gr} (Miller et al., 1976):

$$C = (1 - x)C_{lip} + xC_{Gr}, \quad (6)$$

where x is the fraction of the electrode area covered by the gramicidin. If C_{Gr} is 12–20 μ F cm^{-2} (Miller et al., 1976), which is typical for a polypeptide, then x is $\sim$0.002–0.004. This an order of magnitude higher than the mole fraction of the conducting gramicidin on the electrode

Second, gramicidin is surface active and water insoluble (Kemp and Wenner, 1976). Kemp and Wenner (1976) showed that an aqueous solution of 5×10^{-9} mol dm^{-3} gramicidin exhibited a 50% decrease in fluorescence after 15 min of standing. If it is assumed that there is a similar loss of gramicidin from solution in the experiments in this study and that the gramicidin lost from the solution (at 12.7×10^{-9} mol dm^{-3} electrolyte concentration) partitions into the phospholipid layer, a surface concentration of gramicidin in the layer of 1.134×10^{-11} mol cm^{-2} would ensue. This is $\sim$4% mol fraction of the covered electrode and more than two orders of magnitude higher than the conducting gramicidin concentration. Finally, increases in the reduction current by up to nearly a factor of 10 can be mediated by altering the lipid environment or the solution composition (e.g., see Fig. 4 *c* and Nelson and Bizzotto, 1999) even though the amount of gramicidin added to the solution is the same. In this case it appears that the lipid environment and solution composition changes enable a greater fraction of the gramicidin in the layer to become conducting.

It is proposed, therefore, that the Tl^+ permeation through gramicidin mimics the C_rE_r mechanism because the gramicidin is present in both conducting and nonconducting forms in dynamic equilibrium in the monolayer. The mechanism underlying the current transient proceeds thus as follows. On application of the potential step into the limiting current region of Tl^+ reduction, the equilibrium between the conducting and nonconducting gramicidin is shifted, giving rise to the exponential decrease in reduction current with time constant $\tau = k^{-1}$. Such a shift in equilibrium in response to a voltage step is consistent with the

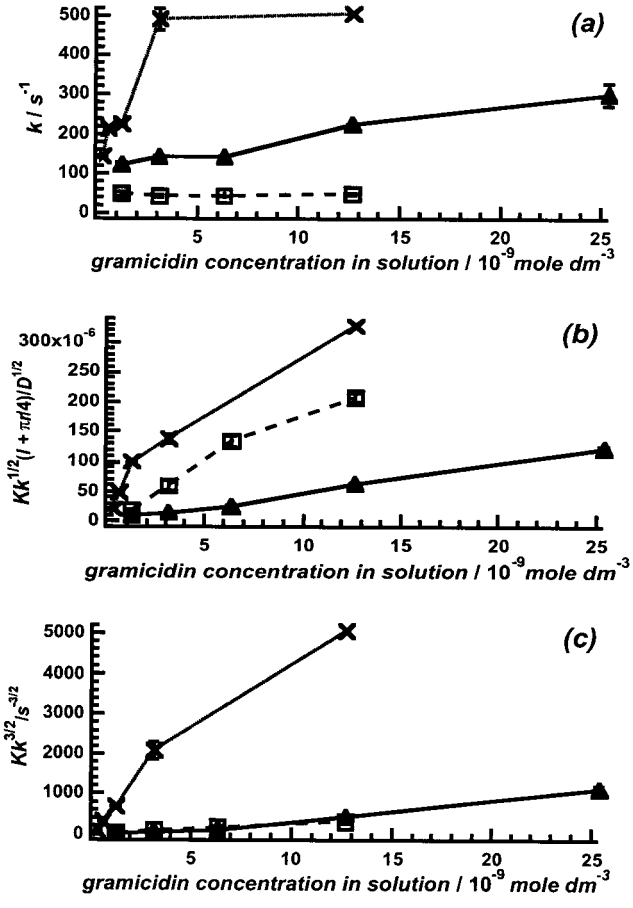

FIGURE 4 Estimated values of k (*a*), $Kk^{1/2}(l + \pi r/4)/D^{1/2}$ (*b*), and $Kk^{3/2}$ (*c*) from potential step experiments at DOPC (▲), DOPC-0.3 PS (in pH 7 electrolyte with 0.001 mol dm^{-3} phosphate buffer; ×), and DOPC-0.12 all-*trans* retinol (□) coated electrodes in electrolyte 0.1 mol dm^{-3} KCl with 10^{-4} mol dm^{-3} Tl^+ and added gramicidin. k, $Kk^{1/2}(l + \pi r/4)/D^{1/2}$ and $Kk^{3/2}$ are plotted versus the gramicidin concentration in solution. Potential step was from -0.2 to -0.55 V.

decrease in gramicidin activity with increase in applied potential in the limiting current region of Tl^+ reduction between potentials -0.5 and -0.7 V (see Fig. 3 *a*). Following the initial current decay, the quasi-steady-state reduction current represents the diffusion of permeant ions to an array of conducting channels in equilibrium with nonconducting channels.

The equilibrium between the nonconducting (Gr) and the conducting forms (Gr*) of the gramicidin molecule can be represented in Scheme 1 below:

$$\mathrm{Gr} \underset{k_-}{\overset{k_+}{\rightleftarrows}} \mathrm{Gr}^*$$

Similar to all current transients through ion channel systems as in Scheme I (Hille, 1992; Colquhoun and Hawkes, 1994), the time constant corresponding to the exponential current relaxation will be equivalent to the reciprocal of the sum of the rate constants, k_+ and k_-; i.e., $\tau = k^{-1} = (k_+ + k_-)^{-1}$. The conducting gramicidin species refers to the $\beta^{6.3}$ helix monomer in the monolayer. The nonconducting inactive forms could be gramicidin aggregates (Naydenova et al., 1995; Ogoshi and Mita, 1997) and/or the double-stranded helix (Dhathathreyan et al., 1988), species that in addition to the $\beta^{6.3}$ helix (Ulrich and Vogel, 1999) have been shown to be present in lipid monolayers. Strictly, k corresponds to the conformational lability of the gramicidin channels. However, if $k_- \gg k_+$ and the concentration of Gr in the monolayer is much higher than that of Gr*, then $k \approx k_-$. In this case the short-time exponential decay of the current transient represents the inactivation of conducting channels.

The heterogeneous rate constant describing the flux of the permeant ion to the channel modified monolayer can thus be written in the following way:

$$k_1^{\mathrm{het}} = (k_+/(k_+ + k_-)) \times N \times \pi r^2 D\mathrm{A_v}/(l + \pi r/4), \quad (7)$$

where $(k_+/(k_+ + k_-))$ is the conducting channel fraction of the total gramicidin (Colquhoun and Hawkes, 1994). N is the surface concentration of total gramicidin in mol cm^{-2} and $\pi r^2 D\mathrm{A_v}/(l + \pi r/2)$ is the ion flux from 1 mol dm^{-3} Tl^+ solution through 1 mol dm^{-3} of channels in the monolayer (Hille, 1992). N can be expressed as $\chi_{\mathrm{Gr}}/\ \pi r^2 A_{\mathrm{v}}$ where χ_{Gr} is the fraction of gramicidin in the monolayer defined as if every molecule in the monolayer occupied the surface area of a conducting channel pore of radius r. Expressed in this way, the surface concentration of a 100% gramicidin-covered electrode is $1/\pi r^2 \mathrm{A_v}$ mol cm^{-2}. Thus, Eq. 7 can be rewritten:

$$k_1^{\mathrm{het}} = (k_+/(k_+ + k_-)) \times \chi_{\mathrm{Gr}} \times D/(l + \pi r/4). \quad (8)$$

In terms of the $\mathrm{C_rE_r}$ analogy, $k_1^{\mathrm{het}} = Kk^{1/2}D^{1/2}$ (Galus, 1993), so the following illustrates the relevance to Scheme 1 of the kinetic factors K and k in Eq. 3:

$$(k_+/(k_+ + k_-)) \times \chi_{\mathrm{Gr}} \times D/(l + \pi r/4) = Kk^{1/2}D^{1/2}. \quad (9)$$

Because $(k_+ + k_-)$ is identified with k in Scheme 1, Eq. 9 can be simplified:

$$k_+ = Kk^{3/2}(l + \pi r/4)/\chi_{\mathrm{Gr}}D^{1/2}. \quad (10)$$

Thus, k_+ is proportional to the experimentally derived $Kk^{3/2}$ at constant χ_{Gr}.

Also,

$$(k_+/(k_+ + k_-)) = Kk^{1/2}(l + \pi r/4)/\chi_{\mathrm{Gr}}D^{1/2}. \quad (11)$$

Rearrangement of Eq. 11 shows that a value for the fractional area of electroactive electrode $(1 - \theta)$ related to the fraction of conducting channels can be estimated as equivalent to $\chi_{\mathrm{Gr}}(k_+/(k_+ + k_-))$; i.e.,

$$\chi_{\mathrm{Gr}} \times (k_+/(k_+ + k_-)) = Kk^{1/2}(l + \pi r/4)/D^{1/2} = (1 - \theta). \quad (12)$$

$1 - \theta$ is also approximately proportional to the quasi-steady-state current at longer time scales and at potentials more negative than -0.5 V, shown in Fig. 1 *a*. Eq. 12 shows that $1 - \theta$ is directly proportional to the stability of the conducting channels (k_+/k_-) when $k_- \gg k_+$.

Influence of lipid environment, solution composition, and applied potential on gramicidin speciation in the monolayer

Aromatic/conjugated compounds

Fig. 2 *b* summarizes the influence of aromatic/conjugated compounds on the fraction of conducting channels in the monolayer as calculated from Eq. 12 using the values of k in Fig. 2 *a*. An increase in the aromaticity of the incorporated compound correlates with an increased fraction of conducting channels in the phospholipid layer. Pyrene compounds in a lipid environment have been shown to associate with the gramicidin tryptophan residues close to the lipid-water interface by Engelke et al. (1994, 1995, 1996). Although their results were observed in a bilayer, similar interactions can be expected in a lipid monolayer. As a consequence, it may be deduced from the results in Fig. 2 *b* that aromatic/conjugated compounds within a lipid monolayer stabilize the gramicidin $\beta^{6.3}$ helix by associating with the tryptophan residues. This is consistent with the fact that the tryptophan residues are critical in maintaining the monomer $\beta^{6.3}$ helix structure (Wallace, 1990, 1996, 1998; Killian, 1992; Seoh and Busath, 1995; Mukherjee and Chattopadhyay, 1994).

Electrolyte alkali metal ions and applied potential

The following refers to equilibrium between the metal ions in bulk solution (K^+ and Tl^+) and the gramicidin species (Gr and Gr*) and gramicidin-metal ion complexes (Gr^*K^+

and Gr^*Tl^+) in the phospholipid phase. Assuming that the gramicidin is speciated in two forms, the ratio of the concentrations of total gramicidin (Gr_T) to conducting gramicidin species in the monolayer can be written taking concentrations as approximately equivalent to activities:

$$([Gr_T]/[Gr^*]) = 1 + ([Gr]/[Gr^*]) + \beta'_{Gr^*K^+}[K^+] + \beta'_{Gr^*Tl^+}[Tl^+] \quad (14)$$

Equation 14 applies similarly to monomolecular channels in monolayers as to bimolecular channels in bilayers (Hinton et al., 1986) and $\beta'_{Gr^*K^+}$ and $\beta'_{Gr^*Tl^+}$ are the stability constants of K^+ and Tl^+ with the monomolecular gramicidin channel in the monolayer. The ratios of the terms $1:([Gr]/[Gr^*]):\beta'_{Gr^*K^+}[K^+]:\beta'_{Gr^*Tl^+}[Tl^+]$ represent the ratios of the concentrations of the species Gr*, Gr, Gr^*K^+, and Gr^*Tl^+ to each other, respectively, in the monolayer.

The stability constants, $\beta''_{Gr^*K^+}$ and $\beta''_{Gr^*Tl^+}$ of K^+ and Tl^+ with the bimolecular gramicidin channel are 52.6 and 582 mol^{-1} dm^3, respectively (Hinton et al., 1986), and it is assumed that the K^+ and Tl^+ affinity for the unimolecular channel in the lipid monolayer is similar to the ions' affinity for the bimolecular channel in bilayers or $\beta'_{GrK+} \approx \beta'_{GrK+}$ and $\beta'_{Gr^*Tl^+} \approx \beta''_{Gr^*Tl^+}$. Accordingly, the metal ion-gramicidin stability constants and the electrolyte concentrations 0.1 and 10^{-4} mol dm^{-3} of K^+ and Tl^+, respectively, can be substituted for the two complexation terms on the right side of Eq. 14. In this way, the ratios of the species' concentrations Gr*, Gr, Gr^*K^+, and Gr^*Tl^+ in the monolayer can be given, respectively, as follows: $-1:([Gr]/[Gr^*]):5.26:0.058$. As a result, the ratio of the concentration of total Tl^+ conducting gramicidin species, $[Gr^*] + [Gr^*Tl^+]$, to the concentration of total Tl^+ nonconducting gramicidin species, $[Gr] + [Gr^*K^+]$ in the monolayer is therefore $-1.058:\{([Gr]/[Gr^*]) + 5.26\}$. If the nonconducting species of gramicidin has a significant concentration and $([Gr]/[Gr^*])$ is $\gg 5.26$, complexation by the univalent ion electrolyte will have a small effect on the concentration of Tl^+ conducting gramicidin in the monolayer. On the other hand, if the major fraction of gramicidin is present as the conducting form and $([Gr]/[Gr^*])$ is very small, then 84% of the conducting channels in the monolayer will be complexed with K^+ and unavailable for Tl^+ conduction. In this case, the K^+ will have a significant effect on Tl^+ conduction. Furthermore, making similar assumptions as above regarding the gramicidin channel ion stability constants, alkali metal ion electrolytes will suppress Tl^+ reduction in the order of the ions' affinity for the bimolecular gramicidin channel, which is $Cs^+ > K^+ > Na^+ > Li^+$ (Wang et al., 1995; Hinton et al., 1986).

The effects of the alkali ions on the Tl^+ reduction current (Fig. 3 *a*) does not follow this order that supports the proposition that $([Gr]/[Gr^*])$ is large and that the conducting species of gramicidin is not the predominant form of gramicidin in the monolayer. Fig. 3 *a* shows that, in fact, Li^+ depresses the Tl^+ reduction current, presumably by lowering the available channel concentration in the monolayer. This effect could be operative at the bulk solution or monolayer level or both. Fig. 3 *b* displays the k vs. $-E$ plot for the reduction of Tl^+ at the gramicidin-modified DOPC-coated electrodes in different electrolytes. These results show that Li^+ in solution decreases the rate of conformational lability of the channels in the monolayer.

*PS and all-*trans *retinol*

It is instructive to compare results obtained with gramicidin-modified DOPC-0.3-PS-coated electrodes within the context of the present discussion. In Fig. 4, *a–c*, are plotted the parameters k, $Kk^{1/2}(1 + \pi r/4)/D^{1/2}$ (the fraction of conducting channels) and $Kk^{3/2}$ (which is proportional to k_+ at a given gramicidin concentration in the monolayer), respectively, versus the gramicidin concentration in solution for three different monolayer systems. The higher currents at the gramicidin-modified DOPC-0.12 all-*trans* retinol electrodes (see Fig. 1 *c*) are correlated with the presence of an increased fraction of conducting channels (see Fig. 4 *b*) owing to a decrease in the value of k (see Fig. 4 *a*). Because the channel activation rate is not increased relative to that of channels in DOPC-coated electrodes (see Fig. 4 *c*), the increase in fraction of the conducting channels is linked to a decrease in the inactivation rate of the channels.

The higher currents at the gramicidin-modified DOPC-0.3-PS-coated electrodes (e.g., Nelson and Bizzotto, 1999) appear to represent an increased fraction of conducting channels in the monolayer (see Fig. 4 *a*) due to an increase in the rate of channel activation (see Fig. 4 *c*). It may be argued that the negative charge on the PS molecule is responsible for increasing the Tl^+ entry and translocation rate into and through the gramicidin channel, respectively. However, the effect of the PS on the gramicidin function increases the rate constant, k, of the initial exponential decay of the current transient (see Fig. 4 *b*), which indicates that the PS is affecting the conformational lability of the gramicidin species in the monolayer.

SUMMARY

The Tl^+ reduction current transients resulting from potential steps applied to a gramicidin-modified phospholipid-coated electrode can be divided into two sections. At short time scales, there is an exponential decay of current. At longer time scales, there is a quasi-steady-state that corresponds to the diffusion of an ion to a bimolecular heterogeneous reaction. The exponential decay of current at short time scales has a time constant from 3 to 25 ms and represents the inactivation of monomer gramicidin channels. Concentrations of conducting gramicidin channels calculated from the quasi-steady-state current are low and together with the initial gramicidin channel inactivation indicate that a pro-

portion of conducting gramicidin is in equilibrium with a larger proportion of nonconducting gramicidin in the layer. Aromatic/conjugated compounds increase the reduction current by decreasing the rate of inactivation of channels and increasing their stability. This effect is positively correlated with the degree of aromaticity. Negatively charged PS increases the reduction current by apparently augmenting the rate of activation of the channels. The effect of the increasing applied potential is to decrease the availability of the conducting channels. The anomalous influence of alkali metal ions on the reduction current is consistent with the model of gramicidin in the monolayer being speciated in more than one form.

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