Gramicidin Channel Kinetics under Tension

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ABSTRACT We have measured the effect of tension on dimerization kinetics of the channel-forming peptide gramicidin A. By aspirating large unilamellar vesicles into a micropipette electrode, we are able to simultaneously monitor membrane tension and electrical activity. We find that the dimer formation rate increases by a factor of 5 as tension ranges from 0 to 4 dyn/cm. The dimer lifetime also increases with tension. This behavior is well described by a phenomenological model of membrane elasticity in which tension modulates the mismatch in thickness between the gramicidin dimer and membrane.

INTRODUCTION

The local lipid environment plays an important role in determining membrane-protein structure and function (Devaux and Seigneur, 1985; Bienvenie and Marie, 1994). However, the relative roles of nonspecific membrane-protein interactions, mediated by bilayer elasticity, versus specific interactions, which depend on the detailed chemistry of the lipids and proteins, have not been elucidated. A variety of theories of nonspecific interactions have been proposed, but there has been relatively little in the way of quantitative measurements to test and refine these models (for reviews see Suckmann, 1983; Abney and Owicky, 1985; Gennis, 1989; Mouritsen and Bloom, 1993; Goulian, 1996).

In this paper we describe the effect of membrane tension on the kinetics of gramicidin A, a channel-forming peptide that can serve as a local probe of bilayer elasticity (Elliott et al., 1981; Sawyer et al., 1989; O’Connell et al., 1990; for reviews see Andersen et al., 1996; Koepp and Andersen, 1996). Gramicidin A is a 15-amino acid hydrophobic peptide that forms cation-selective channels by the transmembrane association of two nonconducting monomers (Bamberger and La¨uger, 1973; Veatch et al., 1975; O’Connell et al., 1990; Cifu et al., 1992) (Fig. 1). Single-channel recording therefore provides a convenient means of monitoring the state of the peptide (i.e., dimer versus monomers). The kinetics of dimer formation and dissociation depends on the interaction between the dimer and membrane. Varying membrane tension modulates the strength of this interaction.

It is often difficult to interpret experiments that probe membrane elasticity at the small length scales relevant to protein structure and function because of the presence of multiple components in the bilayer. Biological membranes are composed of hundreds of different lipids and proteins, and model membrane systems suitable for electrophysiological studies of function often contain varying amounts of hydrocarbons such as decane or squalene, and occasionally charged lipids or cholesterol, to improve membrane stability and adhesive properties. In these systems one needs to consider the local membrane composition, which couples to membrane elasticity. This is an interesting question, which is likely to be important in vivo. However, without concomitant measurements of the local composition, it becomes difficult to interpret the data quantitatively.

We avoid this problem of multiple membrane components by combining pipette aspiration (Kwok and Evans, 1981) and single-channel recording techniques to control membrane tension, while measuring channel kinetics in bilayer vesicles composed of only a single lipid, dioleoyl-phosphatidylcholine (DOPC). We find that the dimer formation rate exhibits a remarkably strong tension dependence, increasing by a factor of 5 as tension ranges from −0 to 4 dyn/cm. The dimer lifetime also increases with tension, but to a lesser extent. We argue that for gramicidin the essential effect of tension is to thin the membrane. Our results are well described by a phenomenological model of bilayer elasticity (Huang, 1986), in which tension changes the thickness mismatch between gramicidin and the bilayer.

MATERIALS AND METHODS

Vesicle preparation

The preparation of vesicles followed a modification of the procedures described by Reeves and Dovehe (1969) and Needham and Evans (1988). Purified [Val1]gramicidin A (VgA) and [Gly1]gramicidin A (GgA) were a gift from R. E. Koepp, II. DOPC dissolved in ethanol (100 mg/ml) (Avanti Polar Lipids, Birmingham, AL) was mixed with a solution of gramicidin dissolved in ethanol (total volume 20–50 µl), deposited on a clean roughened teflon square (~1 cm x 1 cm) in a glass vial, and dried overnight under house vacuum at room temperature. Molar ratios of gramicidin to DOPC were in the range 0.3–1.0 x 10^-7 and 0.6–1.2 x 10^-6 for VgA and GgA, respectively. The vial containing the dried gramicidin-DOPC deposit was loosely capped and placed in a closed plastic box with an excess of water to provide a water-saturated atmosphere and incubated at 47°C for 1 h. A solution consisting of 0.5 M sucrose,
acidiﬁed to pH 2.6 with HCl (1 ml), was then gently added, the cap was tightened, and the sample was further incubated at 47°C for 8 h in the box. A dense dispersion of vesicles was harvested from the cloudy fraction floating near the surface of the sucrose solution (≈100 μl).

With time, DOPC will be hydrolyzed at the low pH (pH 1) at which our measurements were made (see below). We examined the extent of this problem by incubating DOPC suspensions with either 0.1 or 1 M HCl for both 1 and 3 h at room temperature (25–30°C) and examining the reaction mixture by thin-layer chromatography on silica plates (Whatman LK6D; Whatman, Clifton, NJ), using a mobile phase consisting of chloroform: methanol:water (65:45:4 v/v). The detection limit was 10% hydrolysis, determined with serial dilutions of lysophosphatidylcholine. There was no detectable hydrolysis after 3 h of incubation in either 0.1 or 1 M HCl. (The lipids were completely hydrolyzed after 60 h of incubation at room temperature in either 0.1 or 1 M HCl.)

Pipette aspiration and electrical recording

Pipette aspiration and video microscopy were performed on a modiﬁed version of the apparatus described by Elbaum et al. (1996) and Frygenson et al. (1997) (Fig. 2 A). The sample cell consisted of an o-ring (1.8 inch × 7/8 inch) sealed to a 0.17-mm coverslip with parafﬁn. The cell was mounted on a motorized stage on a Zeiss Axiovert 35 inverted microscope (Carl Zeiss, Thornwood, NY), which was equipped with differential interference contrast optics. The temperature of the microscope objective (Zeiss 1.4 NA, Plan Apochromat), which was in thermal contact with the sample cell via immersion oil, was maintained at 23 ± 0.5°C. For illumination, a Zeiss 0.63 NA long-working-distance condenser was coupled to a 75-W xenon lamp (Opti Quip, Highland Mills, NY) via an optical ﬁber scrambler (Technical Video, Woods Hole, MA); critical illumination was resolved. Current records were visualized using our own software, and formation of individual dimers (single-channel events) could be easily traced.

Data analysis

To determine the gramicidin dimer lifetime, we used vesicles with a relatively low gramicidin/lipid ratio (GgA/lipid = 6 × 10⁻⁴), so that the formation of individual dimers (single-channel events) could be easily resolved. Current records were visualized using our own software, and single-channel events were identiﬁed manually. Events that were ambiguous, either because there was more than one conducting channel, or because the single-channel current transitions could not be clearly resolved, were discarded.

The survival probability, P(t), is the probability that a gramicidin dimer that forms at time t = 0 has not dissociated after time t. (The lifetime probability density function is equal to dP/dt.) To calculate P(t), all recorded dimerization events in a single vesicle at ﬁxed tension were taken to open at t = 0. P(t) was then computed from P(t) = N(t)/N(0), where N(t) is the number of dimers that remain after time t, and N(0) is the initial number of dimers. Nonlinear ﬁts to P(t) were performed with Kaleidagraph (Synergy Software, Reading, PA).

To determine the dimer formation rate as a function of tension, we made measurements at different tensions in the same vesicle. GgA/lipid was in the range of 0.6–1.2 × 10⁻⁶. The vesicles were ﬁrst aspirated at a high pressure, and the current was recorded (usually for ~100 s). The pressure was then lowered in increments, with recordings at each pressure level. Attempts to move in increments from low to high pressure were unsuccessful because of vesicle breakdown, adhesion to the glass, or blebbing.

\[ \sigma = \frac{PR_p}{2(1 - R/R_c)} \]
To ensure that the results would not be biased by changes in the vesicle intrusion length $L$ (Fig. 2B), only results for which there was no more than 10% variation in the total current were analyzed. Formation rates were determined with our own software. Briefly, the time derivative of the current was calculated by locally fitting the current versus time to a line with a three- or nine-point fit (depending on the filter cutoff frequency). Points where the slope exceeded a threshold (which also depended on the filter cutoff) were counted as dimer formation events.

RESULTS

The combined pipette aspiration and single-channel recording technique described in this paper entails recording current flow through a micropipette containing an aspirated vesicle, as in Fig. 2B. This leads to two important differences from conventional single-channel recording techniques, in which a single bilayer patch makes a high-resistance seal across a micropipette or hole in a teflon partition. First, for an aspirated vesicle, there is current flow between the glass and the vesicle intrusion. This leak resistance is in parallel with the membrane inside the pipette (inner membrane). Second, current continuity requires that current flowing through the inner membrane must also pass through the membrane outside the pipette (outer membrane), i.e., there is a resistance in series with the inner membrane. Below, we first characterize the leak resistance and give an example of single-channel recordings that are in parallel with the leak. We then show that the resistance of the outer membrane can be neglected.
Vesicles aspirated at a fixed pressure usually drifted into the pipette continuously, but slowly, until they either blebbed or burst. In association with this drift, there was an increase in the leak resistance. Fig. 3 shows a plot of the pipette resistance as a function of intrusion length \( L \) for an aspirated vesicle. We attribute the electrical leak to current flow along the pipette within the aqueous layer between the membrane and the glass (Evans and Rawicz, 1990). Consistent with this interpretation, the pipette resistance is an approximately linear function of \( L \), with a slope of \( \sim 2 \) M\( \Omega/\mu m \). Taking the resistivity of 0.1 M HCl to be 30 \( \Omega \) cm (Robinson and Stokes, 1968), this corresponds to an aqueous layer of thickness 25 nm.

For our measurements, this parallel leak resistance is unavoidable because for the membrane tension to be given by Eq. 1 (which is the reason for using pipette aspiration), there cannot be complete adhesion between the membrane and the glass. Even for intrusions as large as \( L = 50 \) \( \mu m \), the pipette resistance was much smaller than the \( \sim 10\)-G\( \Omega \) resistance of a gramicidin channel (Fig. 3). Nevertheless, for \( L > \sim 10 \) \( \mu m \), the leak was sufficiently quiet that single channels were easily resolved.

Fig. 4 A shows an example of channel recordings in parallel with the leak current. There is a pronounced modulation in the open-channel current. At least part of this modulation is due to diffusion of the gramicidin dimer within the membrane along the length of the pipette. Because the leak current creates a potential gradient running down the pipette, the open-channel current varies as the channel diffuses in this gradient. The variation in channel current makes lifetime measurements difficult, particularly for a long-lived channel such as VgA (lifetime \( \sim 10 \) s), because conducting channels can diffuse into and out of the pipette. We therefore used the glycine-substituted variant [gly\(^1\)]gramicidin A (GgA), which has a shorter lifetime (< \( \sim 1 \) s) (Durkin et al., 1990; Mattice et al., 1995) for kinetic measurements.

Channels in the outer membrane do not interfere with our measurements because the outer membrane resistance is much less than the 10-G\( \Omega \) resistance of a single channel. To show this, we take advantage of the fact that occasionally gigaseals formed with the outer membrane still intact, i.e., the leak resistance jumped to over 100 G\( \Omega \), presumably because of the membrane inside the pipette adhering to the glass. With gigaseals, we obtained single-channel recordings as in Fig. 4 B. Despite the improvement in signal-to-noise, records with gigaseals were not used for analysis because the tension of the membrane could not be determined (Eq. 1 no longer applies). Gigaseals, however, allow us to address the issue of membrane resistance.

As is evident from the current recordings in Fig. 4 B, there was, on average, less than one channel open in the vesicle membrane. Let us assume that the lipid bilayer has negligible conductivity. In this case, the formation of a gramicidin dimer inside the pipette would result in current transitions as in Fig. 4 B only if there were already a dimer present in the outer membrane. If a third channel then opened, in either the inner or outer membrane, the corresponding current transition would be a factor of one-third smaller than the first transition. However, the current transitions do not show this variation in height (Fig. 4 B). Furthermore, if there were no dimer present in the outer membrane, the formation of a dimer in the inner membrane would result in a current jump that would decay to zero with a time constant \( RC \sim 100 \) ms, where \( R \sim 10 \) G\( \Omega \) is the channel resistance and \( C \sim 10 \) pF is the capacitance of the outer membrane for a 10-\( \mu m \)-radius vesicle. We do not see such current jumps, however. We therefore neglect the
resistance of the outer membrane and take the potential of the vesicle interior to be equal to the potential of the solution in the sample cell.

We have obtained a lower bound on the proton conductance of solvent-free DOPC bilayers from the results of one measurement in which a vesicle ruptured and part of the residual membrane formed a 20-GΩ seal several hundred microns up the pipette, where the pipette radius was 1.7 μm (data not shown). This implies a specific membrane conductance of ≤0.5 mS/cm², which gives a resistance of ≥200 MΩ for a 1000-μm² outer membrane. (Only a lower bound can be obtained, because we do not know the magnitude of the seal resistance.)

**Lifetime**

An example of the survival probability $P(t)$ for GgA dimers in a single vesicle at fixed tension is shown in Fig. 5 A. The results are well fit by a sum of two exponentials:

$$P(t) = \left[ b \exp(-t/\tau_1) + (1 - b)\exp(-t/\tau_2) \right]$$

with characteristic times $\tau_1$ and $\tau_2$ differing by an order of magnitude and $b \approx 1/2$, indicating approximately equal numbers of long-lived and short-lived events. The proliferation of short-lived events, which gave rise to the second time scale, did not appear in similar records for the longer lived VgA (lifetime ≈ 10 s) (results not shown). This
suggests that the short time scale is a characteristic of GgA and not due to experimental artifacts, such as membrane flicker against the glass. Subsequent single-channel recordings of GgA in solvent-depleted DOPC-hexadecane bilayers, using a modified tip-dip method (Hanke et al., 1983; Sawyer et al., 1990), also showed two time scales in 0.1 M HCl, but not in CsCl or NaCl (C. Nielsen and O. S. Andersen, unpublished results). We do not understand the basis for this difference, but note that the presence of two time scales appears to be a general property of GgA in thin bilayers in 0.1 M HCl.

The results for the two lifetimes as a function of tension are shown in Fig. 5 B. \( \tau_2 \) exhibits a trend toward increasing lifetime with tension. The trend in \( \tau_1 \) is less clear. A number of factors contribute to the scatter in our results. First, it is more difficult to extract the lifetime of individual dimers at higher tension because the channel formation rate also increases with tension (see below). Second, the vesicles do not survive as long at high tension, making it difficult to obtain a large number of events. Typical numbers of events used to determine lifetimes ranged from 517 to 30, with a median of 56.

### Formation rate

The dimer formation rate, the number of dimers that form per unit of time, depends on the number of gramicidin monomers and therefore is sensitive to the membrane area inside the pipette as well as fluctuations in gramicidin concentration from vesicle to vesicle. We therefore measured formation rates (for GgA) at different tensions in the same vesicle, where we only kept points for which the intrusion length \( L \) (as measured by the leak current) did not

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**FIGURE 5 (A)** Survival probability \( P(t) \) of GgA from measurements in a single vesicle at fixed tension (0.58 dyn/cm), 58 events. The dashed curve is a two-exponential fit (Eq. 2) with \( \tau_1 = 38 \text{ ms}, \tau_2 = 430 \text{ ms}, b = 0.61 \). (B) Lifetimes \( \tau_1 \) and \( \tau_2 \), derived from two-exponential fits, versus membrane tension \( \sigma \). The three different symbols correspond to measurements in three different vesicles.
vary by more than 10%. A large range of tensions could be probed by incrementally lowering the tension, which worked against the tendency of intrusions to drift further into the pipette. An example of data from such a measurement is shown in Fig. 6A, which plots the number of dimers formed per second, \( f \), as a function of tension \( \sigma \). The curve is a quadratic fit to the data: \( \ln f = C_0 + C_1 \sigma + C_2 \sigma^2 \). Based on 12 such plots (12 different vesicles), the average values for \( C_1 \) and \( C_2 \) are \( C_1 = 0.9 \) cm/dyn and \( C_2 = -0.12 \) cm\(^2\)/dyn\(^2\) (for GgA). The coefficient \( C_0 \) is the logarithm of the formation rate at zero tension and is therefore sensitive to the density of gramicidin molecules. Plots of \( C_1 \) versus \( C_0 \) and \( C_2 \) versus \( C_0 \) show no obvious correlations (results not shown). This suggests that the observed saturation in the formation rate at high tension is not a result of depleting the population of gramicidin monomers. Fig. 6B shows a combined plot of all 12 measurements in which each data set has been shifted so as to obtain the best quadratic fit for the combined sets (shown in the solid curve). The coefficients of the quadratic fit are \( C_1 = 0.7 \) cm/dyn and \( C_2 = -0.09 \) cm\(^2\)/dyn\(^2\). For the average over individual fits and from the combined quadratic fit of the aggregate data, we find that the formation rate increases by roughly a factor of 5 as tension ranges from 0 to 4 dyn/cm.

**FIGURE 6** (A) Plot of the logarithm of the formation rate \( f \) of GgA versus tension \( \sigma \) for a single vesicle. The curve is a quadratic fit: \( \ln(f) = -0.8 + 0.9(\text{cm/dyn})\sigma - 0.1(\text{cm}^2/\text{dyn}^2)\sigma^2 \). (B) Combined plot of 12 different measurements of formation rate versus tension (represented by different symbols). Each curve has been shifted by a constant to obtain the best quadratic fit for the combined data (shown in the solid curve). The solid curve is given by \( \ln f = 0.7(\text{cm/dyn})\sigma - 0.08(\text{cm}^2/\text{dyn}^2)\sigma^2 \).
DISCUSSION

For tension to affect channel formation there must be an associated change in membrane area. For example, alamethicin, a different channel-forming peptide, creates pores of varying size, with larger pores favored at higher tensions (Opsahl and Webb, 1994). For gramicidin, on the other hand, the area occupied by the peptide is approximately the same for monomers and dimers. Instead, the tension dependence stems from the thickness mismatch between dimer and membrane. An increase in tension increases the area per lipid and therefore decreases the membrane thickness. This, in turn, changes the elastic stress on the gramicidin dimer.

The hydrophobic thickness of a DOPC bilayer is 27 ± 1 Å (Lewis and Engelman, 1983). Although the corresponding hydrophobic thickness of a gramicidin dimer has not been determined, it is generally believed to be less than the full 26-Å dimer length (Urry, 1972) and has been estimated to be on the order of ~22 Å (Elliott et al., 1983). For a tension $\sigma$, the work performed in forming the dimer, as a result of tension acting directly on gramicidin, will be approximately $\sigma \pi r_o (d - l) \sin \theta$ (Elliott et al., 1983), where $r_o = 10$ Å and $l = 22$ Å are the gramicidin dimer radius and thickness, $d$ is the membrane thickness, and $\theta$ is the angle depicted in Fig. 7 A. Because $\theta \ll 1$, even a tension of 4 dyn/cm will result in mechanical work that is much less than the thermal energy $k_B T$. In addition, for this work to result in an increase in dimer formation rate and lifetime, the angle $\theta$ would have to be negative. It is therefore unlikely that the direct action of tension on gramicidin significantly perturbs the dimerization kinetics.

Tension, however, also acts by thinning the membrane. Because the bilayer is effectively “incompressible” with respect to volume changes (Evans and Hochmuth, 1978; Evans and Needham, 1987), an applied tension $\sigma$ results in a change in bilayer thickness $\Delta d$:

$$\frac{\Delta d}{d_o} = -\frac{\sigma}{K_a} \quad (3)$$

where $d_o$ is the bilayer thickness at zero tension and $K_a$ is the area expansion modulus. For example, for typical values $K_a = 100$–200 dyn/cm, a change in tension of 4 dyn/cm thins the bilayer by 4–2%. The lipid acyl chains adjacent to gramicidin dimers are therefore compressed, and the relative free energy between dimer and monomers, as well as the activation barrier for dimer dissociation, will have components that depend on the membrane-gramicidin hydrophobic mismatch (Huang, 1986; Lundbaek and Andersen, 1994).

The kinetics of dimerization are described by

$$\frac{dN_d}{dr} = k_+ N_m^2 - k_- N_d$$  \quad (4)

where $N$ is the total gramicidin concentration and $N_m$ and $N_d$ are the monomer and dimer concentrations, respectively. The rate constants are expected to take the usual form for an activated process:

$$k_+ = \nu_+ \exp\left[\frac{-(\Delta G^\circ + \Delta G^a)}{k_B T}\right]$$  \quad (5)

$$k_- = \nu_- \exp\left[\frac{\Delta G^a}{k_B T}\right]$$  \quad (6)

where $\Delta G^\circ$ is the free-energy difference between dimer and monomer and $\Delta G^a$ is the activation energy for dimer dissociation (Fig. 7 B). In principle, the microscopic constants $\nu_\pm$ could depend on membrane tension; we expect such an effect to be small, however, and take them to be constant. The average dimer lifetime is equal to $1/k_-$, and the formation rate is given by $f = k_+ N_m^2$. Assuming that $N_m$ is approximately constant (i.e., $N_m \gg N_d$),

$$\ln(f) = \text{constant} - \frac{\Delta G^\circ}{k_B T} + \frac{\Delta G^a}{k_B T}$$  \quad (7)

$\Delta G^\circ$ and $\Delta G^a$ both have components that depend on the elastic deformation of the membrane. Let $E(x)$ be the elastic energy of a membrane containing a gramicidin dimer of hydrophobic thickness $x$. Because the attractive potential giving rise to the gramicidin dimer is due to hydrogen bonds, the peak of the activation barrier for dimer dissociation...
Minimizing Eq. 10 with respect to \( u(r) \) gives

\[
E(l) = K_o \left[ \frac{1}{2} \int d^3r \left( \frac{K_e}{4} \left( \nabla^2 u \right)^2 + \alpha \left( \nabla u \right)^2 + \frac{K_a}{d_o} u^2 \right) \right]
\]

with \( f_0, f_1, f_2 \) dimensionless functions, which can be expressed in terms of Bessel functions (Nielsen et al., manuscript in preparation). The quadratic dependence of \( E \) on \( \sigma \) follows directly from the linear relation between \( d \) and \( \sigma \) and the fact that Eq. 12 is quadratic in \( d - \ell \).

From Eqs. 9–12, we find that the logarithm of the formation rate will depend quadratically on tension: \( \ln(f) = C_0 + C_1 \sigma + C_2 \sigma^2 \). With reasonable values of the parameters, we find good agreement with the fitted values from experiment. For example, with \( l = 22 \) Å, \( d = 27 \) Å, \( r_o = 10 \) Å, \( K_e = 120 \) dyn/cm, \( K_a = 5.6 \times 10^{-12} \) erg, \( \delta = 1 \) Å, and \( s = -0.3 \), we find that \( C_1 = 0.7 \) cm/dyn and \( C_2 = -0.09 \) cm²/dyn², which are the values we obtained from the combined quadratic fit (see above).

**CONCLUSION**

By combining the single-channel recording and pipette aspiration techniques, we have measured gramicidin A dimerization kinetics as a function of tension in a single-component membrane. Both the gramicidin dimer lifetime and formation rate increase with tension, the latter increasing by a factor of 5 as tension ranges from 0 to 4 dyn/cm. This behavior can be understood by using a phenomenological model of membrane elasticity, in which tension thins the membrane and thereby changes the membrane-dimer mismatch in hydrophobic thickness. We find good agreement with the data for reasonable parameter values. Our results demonstrate in a simple system how membrane deformation can modulate protein function. By applying the techniques described here to the study of other membrane proteins under tension, it should be possible to further probe this elusive but important aspect of membrane biophysics.

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