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The Structure of the Gramicidin A Transmembrane Channel

William Veatch

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520

Gramicidin A is a linear polypeptide antibiotic that facilitates the diffusion of monovalent cations across lipid bilayer membranes by forming channels. It has been proposed that the conducting channel is a dimer which is in equilibrium with nonconducting monomers in the membrane. To directly test this model in several independent ways, we have prepared and purified a series of gramicidin C derivatives. All of these derivatives are fully active analogs of gramicidin A, and each derivative has a useful chromophore esterified to the phenolic hydroxyl of tyrosine #11.

Simultaneous conductance and fluorescence measurements on planar lipid bilayer membranes containing dansyl gramicidin C yielded four conclusions: 1) A plot of the logarithm of the membrane conductance versus the logarithm of the membrane fluorescence had a slope of 2.0 ± 0.3, over a concentration range for which nearly all the gramicidin was monomeric. Hence, the active channel is a dimer of the nonconducting species. 2) In a membrane in which nearly all of the gramicidin was dimeric, the number of channels was approximately equal to the number of dimers. Thus, most dimers are active channels and so it should be feasible to carry out spectroscopic studies of the conformation of the transmembrane channel. 3) The association constant for dimerization is more than 1,000-fold larger in a glycerolester membrane with 26 Å-hydrocarbon thickness than in a 47 Å-glycerolester membrane. The dimerization constant in a 48 Å-phosphatidyl choline membrane was 200 times larger than in a 47 Å-glycerolester membrane, showing that it depends on the type of lipid as well as on the thickness of the hydrocarbon core. 4) We were readily able to detect 10^{-14} mole cm^{-2} of dansyl gramicidin C in a bilayer membrane, which corresponds to 60 fluorescent molecules per square μm. The fluorescent techniques described here should be sufficiently sensitive for fluorescence studies of reconstituted gates and receptors in planar bilayer membranes.

An alternative method of determining the number of molecules of gramicidin in the channel is to measure the fraction of hybrid channels present in a mixture of 2 chemically different gramicidins. The single-channel conductance of p-phenylazobenzene-sulfonyl ester gramicidin C (PABS gramicidin C) was found to be 0.68 that of gramicidin A. In membranes containing a mixture of these 2 gramicidins, a hybrid channel was evident in addition to 2 pure channels. The hybrid channel conductance was 0.82 that of gramicidin A. Fluorescence energy transfer from dansyl gramicidin C to diethylamino-phenylazobenzene-sulfonyl ester gramicidin C (DPBS gramicidin C), provided an independent way to measure the fraction of hybrid channels on liposomes. For both techniques the fraction of hybrid channels was found to be 2ad where a^2 and d^2 were the fractions of the 2 kinds of pure channels. This result strongly supports a dimer channel and the hybrid data excludes the possibility of a

William Veatch is now at the Department of Pharmacology, Harvard Medical School, Boston, MA 02115.

tetramer channel. The study of hybrid species by conductance and fluorescence techniques should be generally useful in elucidating the subunit structure of oligomeric assemblies in membranes.

The various models which have been proposed for the conformation of the gramicidin transmembrane channel are briefly discussed.

Key words: gramicidin A, channel, fluorescence energy transfer, membrane fluorimeter, antibiotic, hybrid channels, gramicidin C derivatives

INTRODUCTION

Gramicidin A, a linear polypeptide antibiotic, renders biological membranes and synthetic lipid bilayer membranes permeable to alkali cations and protons (1–4). The amino acid sequence of valine gramicidin A is (5):

CHO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-
1 2 3 4 5 6 7 8
L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$
9 10 11 12 13 14 15

The distinctive features of this amino acid sequence are the alternation of D and L amino acids, the presence of hydrophobic side chains, and the absence of any charged groups. Gramicidin C is a naturally-occurring variant in which L-tryptophan at position 11 is replaced by L-tyrosine.

Gramicidin A induces cation permeability by forming transmembrane channels rather than by acting as a diffusional carrier (6). Synthetic bilayer membranes containing very small amounts of gramicidin A exhibit discrete changes in conductance which arise from the formation and breakdown of individual gramicidin channels (4). The conductance of a single channel depends on the concentration and type of ion but not on the thickness or viscosity of the membrane (6). It has been observed that the membrane conductance is approximately proportional to the square of the amount of gramicidin A added to the aqueous phase, which suggested that the conducting channel is a dimer (7–8). However, this inference is not unequivocal because gramicidin A has such a low solubility in water that it irreversibly adsorbs to the membrane, and so the actual amount incorporated in the membrane is uncertain. The order of the channel-forming reaction has been further studied by a voltage-jump approach (9), which is based on the finding that the number of channels increases as the membrane is thinned by an applied voltage (6). The results of these voltage-jump experiments are consistent with the hypothesis of an equilibrium in the membrane between a nonconducting monomer and a conducting dimer of gramicidin A (9), but these data do not strongly exclude the possibility that channel formation is a trimerization (10). Similar results have also been obtained from an autocorrelation analysis of the conductance fluctuations (10–11).

Clearly the most direct way to characterize the equilibrium between channel and nonconducting gramicidin in the membrane is to measure directly the surface density of gramicidin in the membrane. To this end Veatch and Blout (12) prepared dansyl gramicidin C, which is a highly fluorescent and fully active analog of gramicidin A. The preparation of the 3 gramicidin C derivatives used in this work are described below. First, we will develop the theoretical framework we need to analyze the simultaneous conductance and fluorescence measurements on planar bilayers containing dansyl gramicidin C. Later we will extend our analysis to predict the fraction of hybrid channels in mixtures of 2 different gramicidins. After we have described the results of the hybrid channel experiments,

we will conclude by considering the various models that have been proposed for the conformation of the gramicidin channel.

PREPARATION OF GRAMICIDIN C DERIVATIVES

The 3 gramicidin C derivatives used in this study, shown in Fig. 1, were formed by reacting counter-current purified gramicidin C (the generous gift of Dr. Erhard Gross) with the corresponding sulfonyl chlorides. The reaction mixture was then chromatographed on a Sephadex LH-20 column to separate the gramicidin C derivative from the unreacted gramicidin. The elution profile for dansyl gramicidin C is shown in Fig. 2a (12). The ratio of the dansyl absorbance at 350 nm to the tryptophan absorbance at 290 nm showed that the retarded peak contained 1.0 mole of dansyl per mole of gramicidin C. Fraction CIII was estimated to be 90% pure, and has been further chemically and physically characterized by Veatch and Blout (12). PABS gramicidin C and DPBS gramicidin C are nonfluorescent and were prepared specifically to be used as acceptors of fluorescence energy transfer from dansyl gramicidin C. The details of their preparation are given by Veatch and Stryer (14).

STRATEGY FOR MAKING SIMULTANEOUS FLUORESCENCE AND CONDUCTANCE MEASUREMENTS

Consider the equilibrium between an ion-conducting channel containing n molecules, G_n, and nonconducting monomers, G_1:

$$n\,G_1 \rightleftharpoons G_n \tag{1}$$

$$[G_n] = K[G_1]^n \tag{2}$$

where K is the equilibrium constant. The channel surface density, $[G_n]$ (mole cm^{-2}), is readily calculated from the membrane conductance because we know the single channel conductance. The membrane fluorescence is, to a first approximation, proportional to the total amount of gramicidin on the membrane, $[G_t]$ (mole cm^{-2}), given by:

$$[G_t] = [G_1] + n\,[G_n] \tag{3}$$

Figure 3 is a plot of the solution of equation 2 and equation 3 for the dimer case (n = 2). Log $[G_2]$ is plotted as a function of log $[G_t]$ for various values of the dimerization

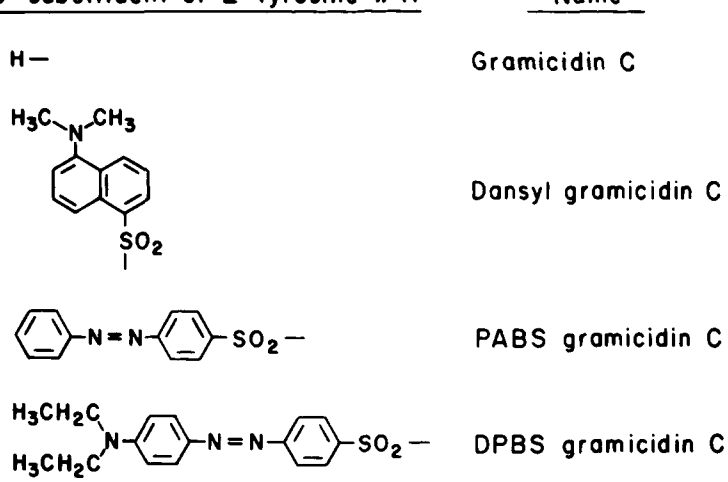

Fig. 1. Structure and nomenclature of derivatives of gramicidin C.

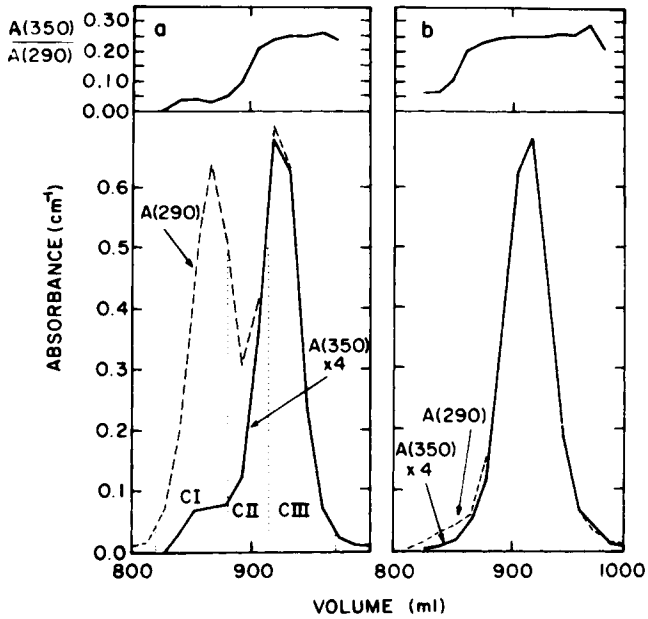

Fig. 2. (a) Elution profile of dansylation products of gramicidin C on Sephadex LH-20 column (150 cm × 4 cm) in methanol. (b) Rechromatography of fraction CIII from (a).

constant K. This solution takes on a simple mathematical form in 2 limiting cases which are of great interest to us. For sufficiently low values of $[G_t]$ and K, essentially all of the gramicidin is monomeric, $[G_t]$ closely approximates $[G_1]$, and the slope of the log-log plot is equal to the number of molecules in the channel, n, here equal to 2.

$$[G_n] \simeq K\,[G_t]^n \qquad \text{for } [G_t]^{n-1}\,K \ll 1 \tag{4}$$

The other limit of great interest occurs when $[G_t]$ and K are sufficiently high so that essentially all of the gramicidin is aggregated. In this case the conductance is not dependent upon the dimerization constant, and the log-log plot has a slope of unity.

$$[G_n] \simeq \frac{[G_t]}{n} \qquad \text{for } [G_t]^{n-1}\,K \gg 1 \tag{5}$$

To test our model and determine n and K for a single membrane, it is evident from Fig. 3 that one would have to measure $[G_2]$ and $[G_t]$ over many orders of magnitude. However, not all combinations of $[G_t]$ and $[G_2]$ are experimentally accessible. $[G_t]$ must be sufficiently high so that the fluorescence of dansyl gramicidin C in the membrane exceeds the background fluorescence due to the membrane lipids. In practice, this occurred when $[G_t]$ was greater than 10^{-14} mole cm^{-2}. In contrast, $[G_2]$ had to be less than about 4×10^{-14} moles cm^{-2} so that the membrane conductance could be measured accurately; at higher values of $[G_2]$, the resistance of the solution surrounding the membrane is much greater than that of the membrane itself. The experimental significance of these limits on the observable values of $[G_t]$ and $[G_2]$ can be appreciated by considering Fig. 3. For a single kind of membrane, it is not feasible to make simultaneous fluorescence and conductance measurements over a range of $[G_t]$ yielding mostly monomer ($[G_t] \ll 1/K$) to mostly dimer ($[G_t] \gg 1/K$). However, these limiting cases, which are most decisive

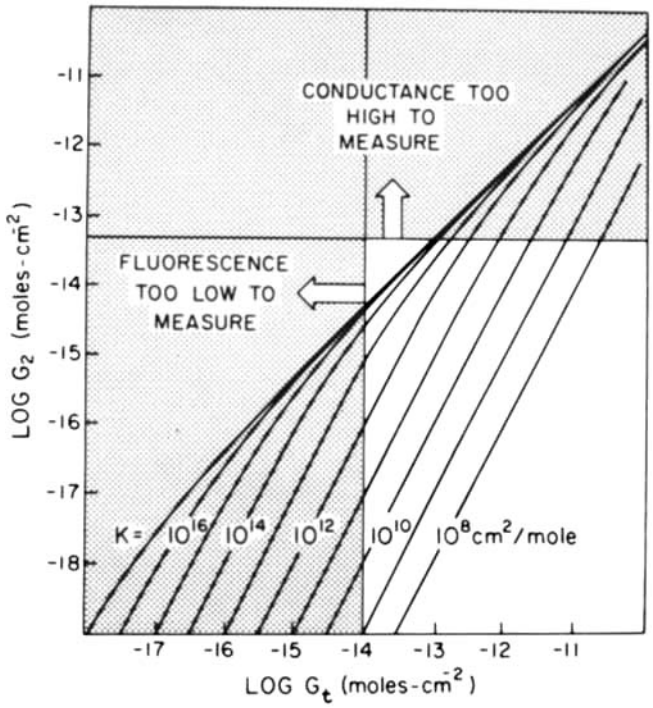

Fig. 3. Theoretical dimerization curves and feasibility region for simultaneous fluorescence and conductance measurements. The curves are plots of the dimer surface density, $[G_2]$, as a function of the total surface density, $[G_t]$, for values of the dimerization constant, K, ranging from 10^8 to 10^{17} mole^{-1} cm^2, according to equations 2 and 3 for n = 2.

in testing the dimer model (equations 4 and 5), are experimentally accessible if membranes having very different values of K are used. This was achieved by using a series of glycerolester membranes differing in the thickness of their hydrocarbon core (6, 13) (Table I). The duration of single channels of gramicidin A in these glycerolester membranes (6) suggested that they have a wide range of dimerization constants, which is in fact the case. We will refer to these membranes by the thickness of their hydrocarbon core (e.g., 26 Å-glycerolester membrane).

Later, we will extend equation 4 and equation 5 to predict the fraction of hybrid channels in mixtures of 2 gramicidins for each of our 2 limiting cases; but first let us consider the results of the simultaneous conductance and fluorescence measurements on planar bilayer membranes containing dansyl gramicidin C.

SIMULTANEOUS CONDUCTANCE AND FLUORESCENCE MEASUREMENTS

Veatch et al. (15) have shown that dansyl gramicidin C is a fully active and fluorescent analog of gramicidin A. The fluorescence excitation and emission spectra of dansyl gramicidin C on liposomes are shown in Fig. 4. The quantum yield was found to be 0.45. The single-channel conductance for dansyl gramicidin C in 1M KCl was found to be 2.4 $\times 10^{-11}$ Ω^{-1}. The membrane fluorimeters used to measure the fluorescence from single planar lipid bilayer membranes, shown schematically in Fig. 5, have been described in detail by Veatch et al. (15).

TABLE I. Hydrocarbon Thickness of Bilayer Membranes Formed From Glycerol Monoester-Alkaline Mixtures

Glycerol monoester	Alkane	Hydrocarbon thickness (Å)
Glycerol-l-palmitoleate	n-hexadecane	26
Glycerol-l-oleate	n-hexadecane	31
Glycerol-l-oleate	n-tetradecane	40
Glycerol-l-oleate	n-decane	47

From Hladky and Haydon (6).

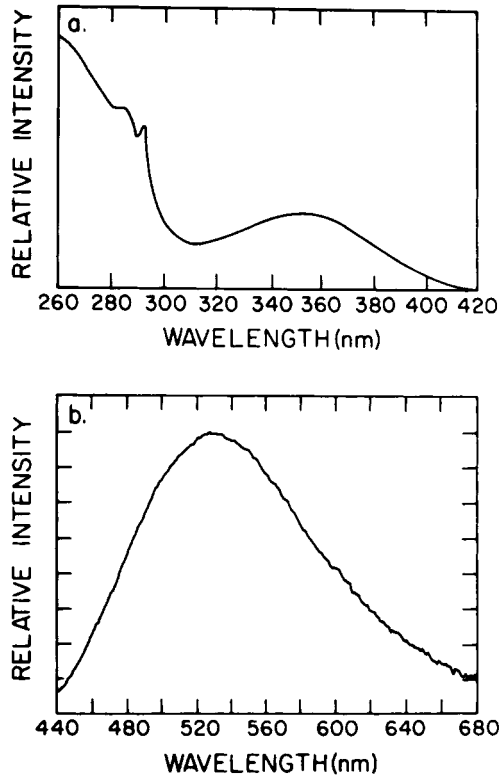

Fig. 4. Corrected fluorescence excitation and emission spectra of dansyl gramicidin C in vesicles. The vesicles contained a 1:50 mole ratio of dansyl gramicidin C to dioleoyl phosphatidyl choline in 0.16 M KCl (plus 4% ethanol). For the excitation spectrum in (a) the emission wavelength was 530 nm and for the emission spectrum in (b) the excitation wavelength was 350 nm. The slit widths were 4 nm and 2 nm, respectively. A red-sensitive photomultiplier tube (EMI 9658R) was used to obtain these spectra.

The conductance and fluorescence intensity of planar bilayer membranes containing dansyl gramicidin C were measured simultaneously to test directly the dimer model. As mentioned previously, such simultaneous measurements are feasible only if the fluorescence is sufficiently high and the membrane conductance is sufficiently low (Fig. 3). We found that simultaneous fluorescence and conductance measurements could be

made over a 10-fold range in the concentration of dansyl gramicidin C in a thick 47 Å-glycerolester membrane. A plot of the logarithm of the conductance versus the logarithm of the fluorescence intensity (Fig. 6) has a slope of 2.0 ± 0.3. For a certain amount of dansyl gramicidin C fluorescence, the 47 Å-glycerolester membrane has about 10^3 lower conductance than the 26 Å-glycerolester membrane. Since gramicidin has the same single-channel conductance on all glycerolester membranes (6), this means that fewer than one gramicidin molecule per 1,000 is involved in channels under these conditions at any given time. Since $[G_t]\ K \ll 1$, the observed slope of 2.0 proves that the channel is a dimer of the nonconducting species.

Is Every Gramicidin Dimer a Conducting Channel?

The absolute surface density of gramicidin must be known to answer this question. Our task of converting the observed fluorescence intensity to the absolute surface density of gramicidin has been greatly facilitated by the work of Zingsheim and Haydon (16). They determined the surface density of 8-anilino-1-napthalene-sulfonic acid (ANS) adsorbed to a 47 Å-glycerolester planar bilayer membrane as a function of the concentration

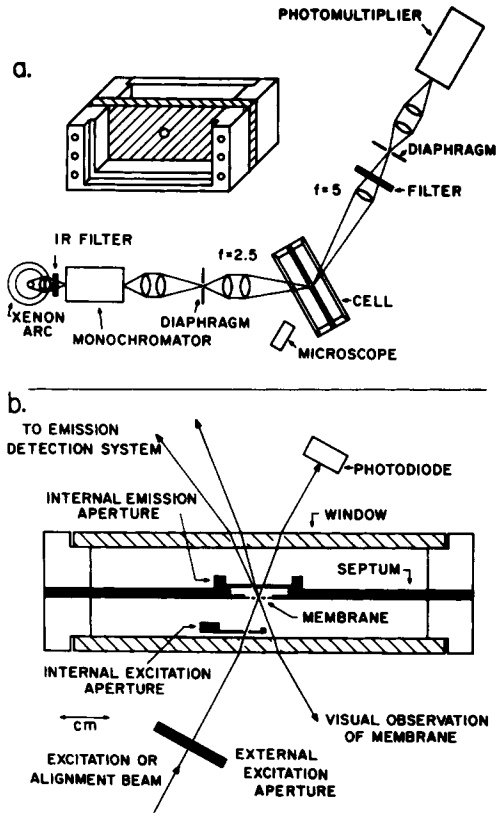

Fig. 5. Schematic diagram of the membrane fluorimeter. Part (a) shows a top view of the physical layout of the standard membrane fluorimeter with a xenon lamp and a monochromator as the excitation source. Part (b) shows a cross-section of a modification of the standard membrane fluorimeter in which an argon-ion laser was used with apertures inside the cell to eliminate the possibility of detecting torus fluorescence. The emission optics external to the cell were unchanged.

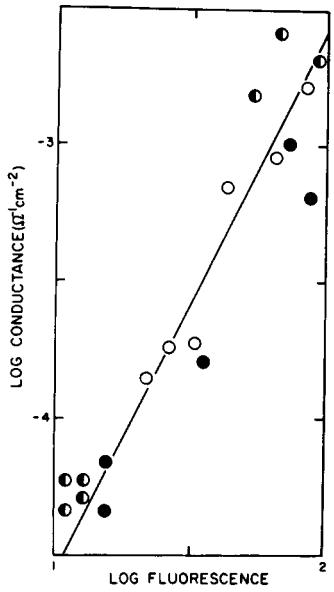

Fig. 6. Conductance as a function of fluorescence for dansyl gramicidin C in 47 Å-glycerolester membranes. The laser excitation modification was used to obtain 3 independent sets of points. These fluorescence values were then normalized using membrane-bound ANS as a fluorescence standard. The least-square slopes for these 3 sets of data are 2.0 ± 0.2 (open circles), 2.1 ± 0.2 (half-filled circles), and 1.7 ± 0.2 (filled circles).

of ANS in the ambient solution. We used a 47Å-glycerolester membrane in 0.1 M NaCl containing 1.3×10^{-6}M ANS, which gives an ANS surface density of 7.1×10^{-13} mole cm^{-2} (16), as a membrane fluorescence standard. The surface density of dansyl gramicidin C could then be calculated from the ratio of the fluorescence intensity of dansyl gramicidin C to that of the ANS membrane standard, taking into account the relative fluorescence parameters of the 2 chromophores (15).

The simultaneous conductance and fluorescence data for membranes of varying thickness are shown in Fig. 7, superimposed upon the calculated dimerization curves of Fig. 3. Note that the fluorescence has been converted to total surface density (mole cm^{-2}) and the conductance to channel surface density (multiplied by 2). For a fixed gramicidin surface density, the membrane conductance increases dramatically as the thickness of the glycerolester membrane decreases from 47 Å (open circles) to 40 Å (half-filled circles), to 31 Å (open square) and 26 Å (filled circle). The estimated dimerization constant, summarized in Table II, increases at least 1,000-fold for a thickness change from 47 Å to 31 Å. However, the structure of the lipid also affects the dimerization constant. The 48 Å thick phosphatidyl choline membrane (filled square) has a dimerization constant 200-fold greater than the glycerolester membrane of the same thickness.

The last important conclusion to be drawn from Fig. 7 requires a quantitative comparison of the fluorescence and conductance found for the 26 Å-glycerolester membrane. The average fluorescence value was about 30% less than that calculated from the average conductance value, assuming that all of the gramicidin on this thinnest membrane was in dimer channels. Because it is estimated that the gramicidin surface density calculated from

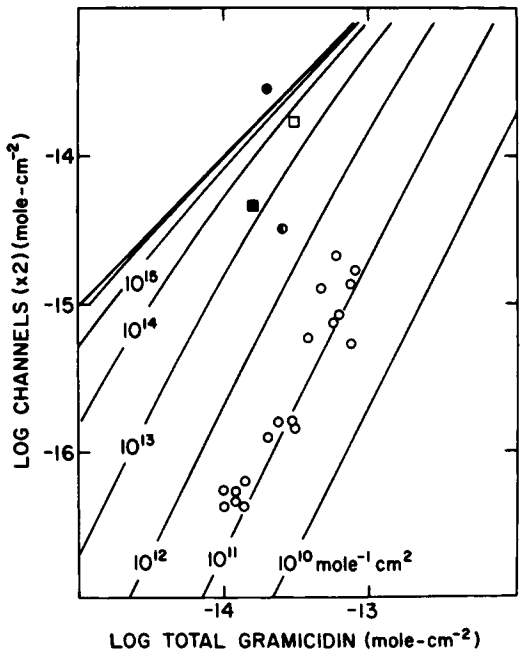

Fig. 7. Channel surface density as a function of total gramicidin surface density for planar bilayer membranes of different composition. The lines are theoretical dimerization curves like those in Fig. 3. The fluorescence has been converted to total gramicidin surface density, and the specific conductance has been converted to channel surface density. The single-channel conductance of the glycerolester membranes in 0.1 M NaCl was taken to be $4 \times 10^{-12}\Omega^{-1}$. For the phosphatidyl choline membrane in 1 M NaCl, the single-channel conductance was estimated to be $7 \times 10^{-12}\Omega^{-1}$. The laser excitation modification was employed for the 40 Å- and the 47 Å-glycerolester membranes and the standard membrane fluorimeter for the others. Open circles: 47 Å-glycerolester membrane; half-filled circle: 40 Å-glycerolester membrane; open square: 31 Å-glycerolester membrane; filled circle: 26 Å-glycerolester membrane; filled square: 48 Å-phosphatidyl choline membrane.

the fluorescence is correct within a factor of 2, these results strongly suggest that most, if not all, gramicidin dimers are conducting channels. Thus it should be possible to use spectroscopic techniques to study the structure of the dimer channel.

THE HYBRID STRATEGY FOR STUDYING OLIGOMERS

An alternative way to determine the number of gramicidin molecules in the transmembrane channel is to measure the fraction of hybrid channels in a mixture of 2 chemically different gramicidins. Figure 8 illustrates the result expected for a dimer. Let us denote the 2 gramicidins as "A" and "D". If the fraction of pure A dimers is a^2 and the fraction of pure D dimers is d^2, then the expected fraction of hybrid dimers is 2ad. This is exactly the result obtained for the gramicidin channel. The results expected for the most simple trimer and tetramer models are summarized in Table III. Veatch and Stryer (14) have used 2 independent methods to measure the fraction of hybrid channels: single-channel conductance measurements and fluorescence energy transfer measurements on liposomes.

TABLE II. Dimerization Constants for Dansyl Gramicidin C Estimated From Simultaneous Fluorescence and Conductance Measurements

Membrane	Hydrocarbon thickness (Å)	Dimerization constant ($mol^{-1} cm^2$)
Glycerolester	47†	1×10^{11}
	40†	3×10^{12}
	31†	$\geqslant 10^{14}$
	26†	$\geqslant 10^{14}$
Phosphatidyl choline	48‡	2×10^{13}

†From Hladky and Haydon (6).
‡From Bamberg and Läuger (9).

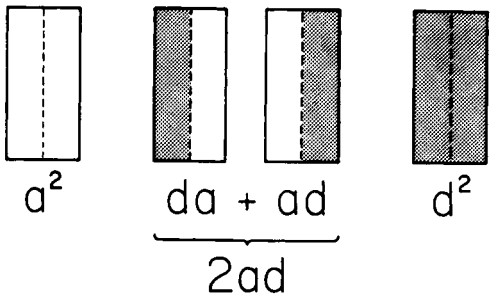

Fig. 8. In the dimer model, the relative proportion of the hybrid species is 2ad, where a and d are the mole fractions of the 2 kinds of gramicidin.

TABLE III. Calculated Probabilities of Pure and Hybrid Channels for Various Models

	Pure A	Hybrid	Pure D
Dimer	a^2	2ad	d^2
Trimer	a^3	$3a^2d + 3ad^2$	d^3
Tetramer	a^4	$4a^3d + 6a^2d^2 + 4ad^3$	d^4
n-mer	a^n	$(a + d)^n - a^n - d^n$	d^n

Single-Channel Conductance Measurements: Demonstration of Hybrid Channels

The single-channel conductance of PABS gramicidin C is 0.68 that of gramicidin A (Figs. 9a, b). An interesting result was obtained for membranes containing both PABS gramicidin C and gramicidin A. These membranes exhibited 3 kinds of channels: a pure PABS gramicidin C channel, a pure gramicidin A channel, and a hybrid channel with an intermediate conductance (Fig. 9c). A histogram of the frequency of occurrence of a conductance step as a function of the step size, measured from many records like the one in Fig. 9c, is shown in Fig. 10. The average single-channel conductance of the hybrid species is 0.82 that of gramicidin A. This value for the hybrid corresponds to the geometric mean of the values for the 2 pure species. The standard deviation of the single-channel conductance of the hybrid is not appreciably greater than that of the 2 pure channels. Thus, the hybrid channel appears to be a unique species.

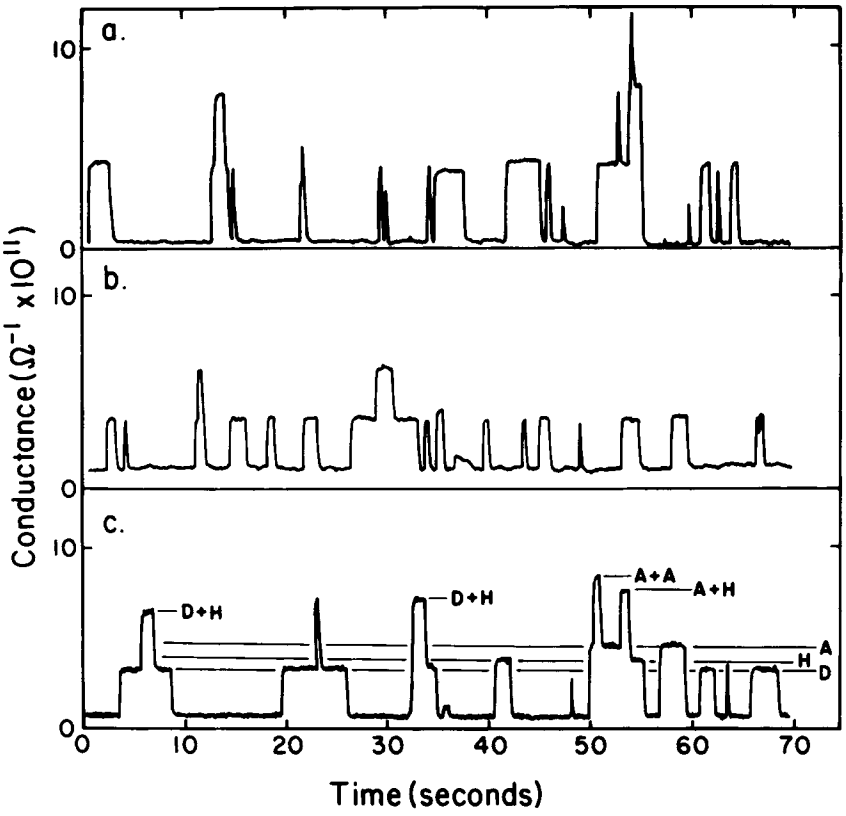

Fig. 9. Single-channel conductance fluctuations of membranes containing (a) gramicidin A, (b) PABS gramicidin C, and (c) a mixture of the 2. The horizontal lines in (c) indicate the conductance corresponding to pure PABS gramicidin C channels (labeled D), pure gramicidin A channels (labeled A) and hybrid channels (labeled H). The conductance levels arising from 2 channels are also shown.

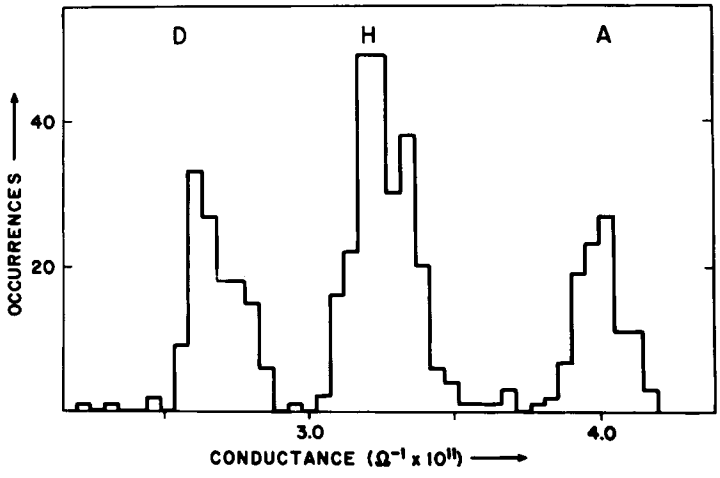

Fig. 10. Frequency of occurrence versus size of the conductance step in membranes containing a mixture of PABS gramicidin C and gramicidin A. The labels D and A refer to the pure species and H to the hybrid. The standard deviation is 2.8% for H, compared to 3.0% for D and 2.3% for A.

The frequencies of these 3 kinds of channels depended on the relative amounts of PABS gramicidin C and gramicidin A in the membrane-forming solution (Fig. 11). The frequency of the hybrid channel reached a maximum value of 0.5 when the frequencies of the pure channels were each about 0.25. This finding suggests that the hybrid channel is a dimer, which is predicted to have a hybrid frequency of $2ad = 0.5$ when $a^2 = d^2 = 0.25$. In the following discussion we will denote the concentrations of pure gramicidin A dimers by [AA], of the pure PABS gramicidin dimers by [DD], and of the hybrid dimers by [AD].

Conductance Evidence That the Hybrid Channel is a Dimer

Because single-channel conductance measurements are made at very low surface densities, only a minute fraction of the gramicidin on these membranes will form channels at any given time. We need to extend our previous expression for the channel surface density in this limiting case, equation 4, rewritten below for $n = 2$:

$$[G_2] \simeq K[G_t]^2 \quad \text{for } K[G_t] \ll 1 \tag{4'}$$

Let us denote the fraction of pure A channels by f_A, the fraction of pure D channels by f_D, and the fraction of hybrid channels by f_H.

$$f_A = a^2 \tag{6}$$
$$f_D = d^2 \tag{7}$$
$$f_H = 2ad \tag{8}$$

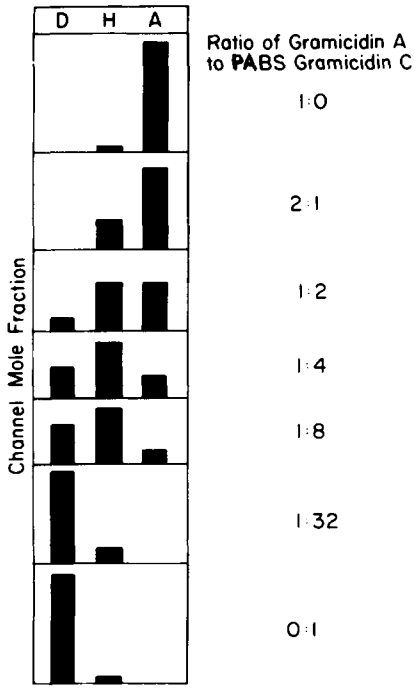

Fig. 11. Probabilities of the 3 kinds of channels (D, H, and A) as a function of the ratio of gramicidin A to PABS gramicidin C in the membrane-forming solution. Each histogram represents the average of several records accumulated by the probability-distribution analyzer (Saicor model 42A).

To understand the meaning of a and d in general, we must define a few more experimental parameters. Let us denote the pure A dimerization constant as K_A, the pure D dimerization constant as K_D, and the hybrid dimerization constant as K_H. The total monomer concentrations in the membrane-forming solution, denoted $[A_s]$ and $[D_s]$, are related to the total monomer surface densities $[A_t]$ and $[D_t]$ by empirical partition coefficients P_A and P_D. Note that all we can determine in this experiment are the concentrations in the membrane-forming solution $[A_s]$ and $[D_s]$, because we can not make fluorescence measurements at these very low surface densities. If the dimerization constants are all equal, and the partition coefficients are both equal as well, then a and d are the mole fractions calculated from the known concentrations in the membrane-forming solution, $[A_s]$ and $[Ds]$, and $a/d = [A_s]/[D_s]$. In general (as long as $K_H^2 = K_D K_A$) the ratio $a/d = \gamma [A_s]/[D_s]$ where

$$\gamma = \left[\frac{K_A P_A^2}{K_D P_D^2}\right]^{1/2} \tag{9}$$

The experimental results can be compared with predictions for the dimer model by casting equations (6) to (8) into a simple form. The ratio of the probability of each of the pure channels to that of the hybrid is directly proportional to the concentration of the 2 gramicidins in the membrane-forming solution.

$$\frac{f_H}{2f_D} = \frac{2f_A}{f_H} = \gamma \frac{[A_s]}{[D_s]} \tag{10}$$

A plot of $\log [f_H/(2f_D)]$ and of $\log (2f_A/f_H)$ versus $\log ([A_s]/[D_s])$ is shown in Fig. 12. Three conclusions can be drawn from these data. First, the experimental data for the ratio of $2f_A/f_H$ are approximately equal to those for $f_H/2f_D$, confirming the dimer model (equation 10). Second, the fit of these data to a line having a slope of 1.0 (the solid line in Fig. 12) indicates that the ratio of the 2 kinds of gramicidin in the membrane is in fact proportional to their ratio in the membrane-forming solution. This proportionality holds at low surface densities, but not at very high surface densities of gramicidin (15). Third, Fig. 12 shows that the factor γ in equation 10 is equal to 3.6. It is known that the dimerization constant for dansyl gramicidin C is not less than that for gramicidin A (15). Most likely, γ differs from unity because gramicidin A partitions more into the membrane than does PABS gramicidin C.

Another way of depicting the experimental data is shown in Fig. 13. The fractions of the 3 kinds of channels are plotted as a function of the effective mole fraction of gramicidin A, which is equal to $\gamma [A_s]/(\gamma[A_s] + [D_s])$. This quantity was calculated from $[A_s]$ and $[D_s]$ using the value of $\gamma = 3.6$ determined from Fig. 12.

Energy Transfer Evidence That the Hybrid Channel is a Dimer

Fluorescence energy transfer was used as a complementary means of ascertaining the frequency of hybrid channels. The strategy was to label one gramicidin with a fluorescent energy donor and another with a suitable energy acceptor. The donor-acceptor pair was chosen so that most of the donor fluorescence in a hybrid channel was quenched by energy transfer to the acceptor. The fluorescence intensity of the membranes containing both kinds of gramicidins then revealed the frequency of hybrid channels, which could be compared with predictions for various models.

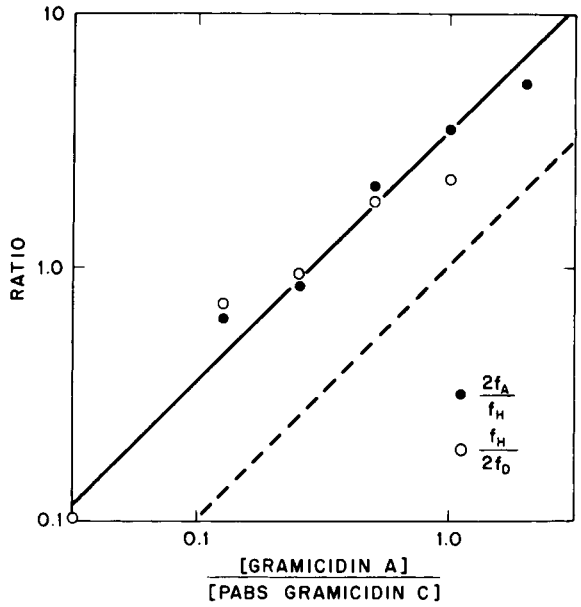

Fig. 12. The probabilities of the 3 kinds of channels (f_A, f_D, and f_H) are shown as a function of the ratio of gramicidin A to PABS gramicidin C in the membrane-forming solution. The ordinate is the ratio $2f_A/f_H$ (filled circles) or $f_H/2f_D$ (open circles). The approximate equality of these ratios supports the dimer model. The fit of these data to the solid line having a slope of 1.0 indicates that the ratio of the 2 kinds of gramicidin in the membrane is proportional to their ratio in the membrane-forming solution. The dashed line is calculated for the case in which the ratio of the 2 gramicidins in the membrane is the same as in the membrane-forming solution. The shift of the solid line from the dashed one indicates that gramicidin A partitions preferentially into the membrane by a factor of 3.6.

Dansyl gramicidin C (Fig. 1) was the fluorescent energy donor in these experiments. PABS gramicidin C (Fig. 1) was considered as a potential energy acceptor. However, it effectively quenches the dansyl fluorescence only at distances of less than about 20 Å because its longest wavelength absorption band is weak (ϵ = 500 $cm^{-1}M^{-1}$ at λ_{max} = 460 nm). The strength of this band was increased by preparing DPBS gramicidin C (Fig. 1), the diethylamino derivative of PABS. The resulting extinction coefficient of 33,000 $cm^{-1}M^{-1}$ markedly extended the range of efficient energy transfer, as calculated below. The overlap of the emission spectrum of dansyl gramicidin C and the absorption spectrum of DPBS gramicidin C is shown in Fig. 14.

In Förster's theory, the efficiency of singlet-singlet energy transfer E is:

$$E = r^{-6} / (r^{-6} + R_0^{-6}) \quad (11)$$

where r is the distance between the centers of the donor and acceptor chromophores, and R_0 is the distance at which the transfer is 50% efficient (17, 18). R_0 can be calculated from

$$R_0 = (Q_0 J n^{-4} K^2)^{1/6} \times 9.7 \times 10^3 \text{ Å} \quad (12)$$

where Q_0 is the quantum yield of the donor in the absence of the acceptor, J is the spectral overlap integral, n is the refractive index of the intervening medium, and K^2 is the dipole-dipole orientation factor. For transfer from dansyl gramicidin C to DPBS gramicidin C, Q_0 = 0.45 (15), J = 5.1 × 10^{-14} $cm^{-3}M^{-1}$ (Fig. 14), and n is about 1.4. K^2 is

assumed to have a value of 2/3, which corresponds to rapid randomization of the orientations of the donor and the acceptor. The plausibility of this assumption is supported by examination of molecular models and by our nanosecond emission anisotropy measurements which show that the dansyl chromophore rotates over an angular range of about 25° in a time that is short compared to the excited state lifetime (19). These values give an R'_0 (20) of 39 Å for transfer from dansyl to DPBS.

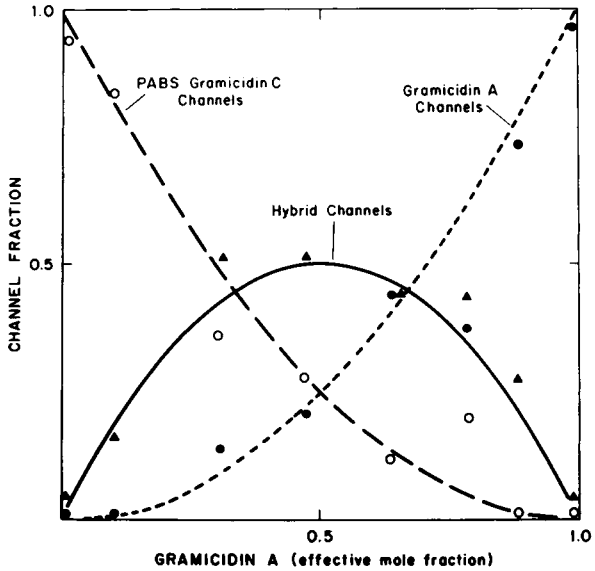

Fig. 13. The observed probabilities of the pure PABS gramicidin C channel (open circle), the pure gramicidin A channel (filled circle), and the hybrid channel (triangle) are plotted as a function of the effective mole fraction of gramicidin A. The calculated probabilities for the dimer model are shown by the dashed, dotted, and solid lines, respectively.

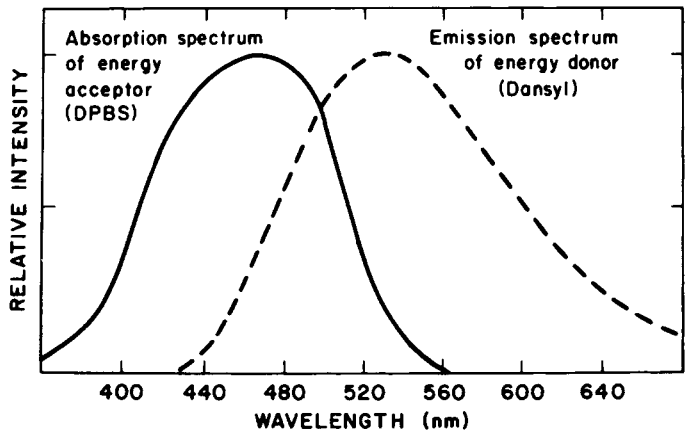

Fig. 14. The absorption spectrum of DPBS gramicidin C (in methanol) overlaps the corrected fluorescence emission spectrum of dansyl gramicidin C (excited at 350 nm) on phosphatidyl choline liposomes. The maximum extinction coefficient of DPBS is 33,000 $cm^{-1}M^{-1}$. The spectral overlap integral is 5×10^{-14} $cm^{-3}M^{-1}$.

Liposomes rather than planar bilayer membranes were used because more precise measurements of the fluorescence intensity of liposomes can be made. Also, the ratio of the mole fractions of the donor and acceptor gramicidins in the membranes of liposomes is in fact equal to the ratio at which they were added. Nearly all of the gramicidin is in the channel form in bilayer membranes having sufficiently short hydrocarbon chains and a high enough surface density of gramicidin (15). These 2 conditions are met in the liposomes used in this study. Hence, our fluorescence energy transfer measurements provide information about the structure of the channel.

Energy transfer measurements were carried out on di-(dihydrosterculoyl) phosphatidyl choline liposomes containing a fixed amount of dansyl gramicidin C and a variable amount of DPBS gramicidin C. The surface density of the total gramicidin was less than 1 gramicidin per 1,000 lipid molecules, which means that the mean distance between channels was greater than 250 Å, and so energy transfer between chromophores in different channels was negligible. As the mole fraction of DPBS gramicidin C, the energy acceptor, was increased from 0 to 0.8, the relative fluorescence quantum yield of the dansyl energy donor decreased from 1 to 0.4 (Fig. 15). This quenching of the dansyl fluorescence is due to efficient energy transfer from dansyl to DPBS within a hybrid channel. No quenching would have been observed if the gramicidins were monomeric in this membrane.

We will denote the pure dansyl gramicidin C dimers by [DD], the pure DPBS gramicidin C dimers by [AA], and the hybrid by [AD], where here D refers to the energy donor and A to the energy acceptor. Let us derive the dependence of Q/Q_0, the relative fluorescence quantum yield of the donor, on a, the mole fraction of the energy acceptor, for a dimer. First, we need to extend our previous expression for the channel surface density in the limiting case where almost all of the gramicidin is dimerized, equation 5, rewritten below for n = 2:

$$[G_2] \simeq \frac{[G_t]}{2} \qquad \text{for } K\,[G_t] \gg 1 \tag{5'}$$

We again obtain the results summarized in equations 6–8. Here a and d are the mole fractions calculated from the total membrane surface densities, $[A_t]$ and $[D_t]$, which we know directly on liposomes. These results are independent of any difference between the dimerization constants K_A and K_D (so long as $K_H^2 = K_D K_A$) because essentially all of the gramicidin is dimerized.

If the transfer efficiency within a hybrid dimer is E, then Q/Q_0 is

$$\frac{Q}{Q_0} = \frac{2[DD] + (1 - E)\,[AD]}{2[DD] + [AD]} = 1 - \frac{E[AD]}{2[DD] + [AD]} \tag{13}$$

Substituting equations 6, 7, and 8 into equation 13, one obtains the very simple expression for the quenching in a dimer

$$Q / Q_0 = 1 - E\,a \tag{14}$$

Thus, for a dimer channel, the relative quantum yield of the donor decreases linearly with the mole fraction of the acceptor. For the simplest trimer models in which all hybrid species are assumed to have the same transfer efficiency (see Table III), $Q/Q_0 = 1 - E + E\,(1 - a)^2$, and for the simplest tetramer model, $Q/Q_0 = 1 - E + E\,(1 - a)^3$.

The observed dependence of Q/Q_0 on a is shown in Fig. 15 with the least-squares fits of the dimer, trimer, and tetramer model to these data. The experimental results best

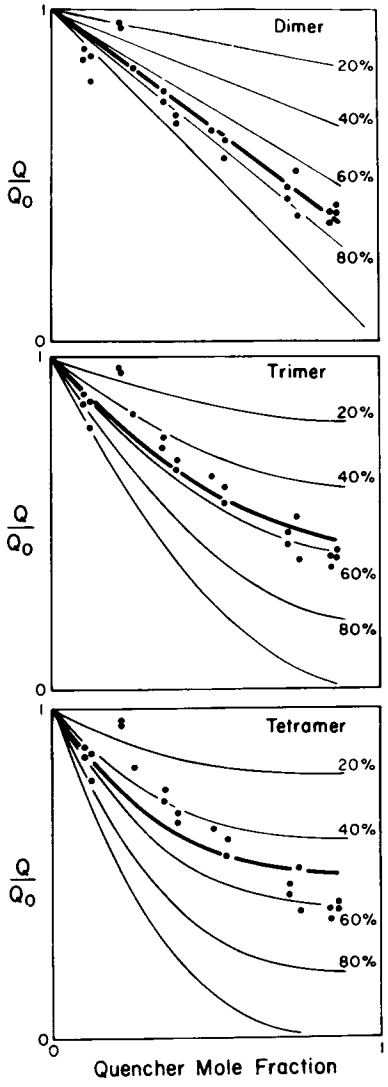

Fig. 15. Relative fluorescence quantum yield of dansyl gramicidin C, the energy donor, as a function of the mole fraction of DPBS gramicidin C, the energy acceptor. The experimental data (closed circles) are compared with light curves calculated assuming 20%, 40%, 60%, 80%, and 100% energy transfer for (a) dimer, (b) trimer, and (c) tetramer models. The bold curves are the least-squares best fits. The best fit standard deviation for the tetramer model is 90% greater than for the dimer model.

fit a dimer model (Fig. 15a). The trimer model cannot be excluded by these results alone (Fig. 15b). However, the trimer model is incompatible with the results of the simultaneous conductance and fluorescence study, which requires that the number of molecules in the channel be an even number, since we proved that the channel was a dimer of the non-conducting species. The tetramer model can be rejected in view of the poor fit of the experimental data to this model (Fig. 15c). The least-squares fit to the dimer model (Fig. 15a) shows that the efficiency of energy transfer within the hybrid channel is 75%. The apparent distance between the centers of the dansyl and DPBS chromophores is 33 Å for $E = 75\%$ and $R'_0 = 39$ Å.

DISCUSSION

Simultaneous Conductance and Fluorescence Measurements on Planar Bilayers

The structure of the gramicidin transmembrane channel is of interest as a model for the passive ion-pathways of cell membranes. The chromophores of the gramicidin C derivatives (Fig. 1) provide useful probes of that structure. The conductance properties of all of these derivatives are very similar to those of gramicidin A. The strong fluorescence of dansyl gramicidin C enabled us to measure its absolute surface density in planar bilayer membranes. In previous studies, the concentration of gramicidin A in the bilayer membrane was not directly measured. The number of active channels corresponding to a particular surface density of gramicidin was ascertained from simultaneous measurements of the membrane conductance. Three conclusions can be drawn from these experiments:

1) The slope of 2.0 ± 0.3 in the double logarithmic plot of the conductance versus the fluorescence intensity over a concentration range for which nearly all of the gramicidin is nonconducting (Fig. 6) proves that the channel is a dimer of the nonconducting species.

2) In the 26 Å-glycerolester membrane, nearly all of the dansyl gramicidin C was dimeric. The number of channels in this membrane was approximately equal to the number of dimers. Hence, not only are all the channels dimers, but all the dimers are channels.

3) The dimerization constant of dansyl gramicidin C increases 30-fold in going from a 47 Å- to a 40 Å-glycerolester membrane (Table II). The dimerization constant in a 48 Å-phosphatidyl choline membrane is 200 times larger than in a 47Å-glycerolester membrane. Thus, the dimerization constant depends markedly on both the type of lipid and on the thickness of the hydrocarbon core. Hladky and Haydon (6) found that the mean duration of a single channel increases monotonically the thinner the membrane. They attributed this effect to a variation in the extent of local thinning or dimpling of the membrane in the vicinity of the conducting channel. Our data are consistent with their proposal. The greater than 1,000-fold decrease in the dimerization constant in going from a 31 Å- to a 47 Å-glycerolester membrane corresponds to an increase in the free energy of dimerization of at least 4.5 kcal/mole. This free energy difference might represent the cost of deforming a small region of the 47Å membrane to match the length of the gramicidin channel.

We can detect the fluorescence of as few as 50 molecules per square μm on planar bilayer membranes. This surface density is comparable to that of many receptors in vivo. If these receptors can be reconstituted into planar membranes at these surface densities, then they can be studied using the fluorescence techniques which we have developed.

Single-Channel Conductance and Energy Transfer Measurements of Hybrid Channels

Our single-channel conductance and fluorescence energy transfer experiments show that hybrid channels are formed when two kinds of gramicidins are mixed. These hybrid channels differ from the pure species in 2 readily observable ways. First, hybrid channels have a single-channel conductance intermediate between those of the pure species. The relative conductance of the hybrid channel is 0.82, whereas those of pure PABS gramicidin C and of the pure gramicidin A channel are 0.68 and 1.00, respectively. Second, the efficiency of energy transfer between dansyl gramicidin C, the energy donor, and DPBS gramicidin C, the energy acceptor, in a hybrid channel is 75%. There is virtually no energy transfer between different channels if the surface density of gramicidin in the membrane is sufficiently low. In both the conductance and energy transfer experiments, the quantitative

dependence of the fraction of hybrid channels on the ratio of the 2 kinds of gramicidin in the membrane reveals that the channel consists of 2 molecules of gramicidin. In particular, the possibility of a tetrameric channel of gramicidin in equilibrium with a nonconducting dimer is excluded by these hybrid studies.

The study of hybrid species should be generally useful in elucidating the subunit structure of oligomeric assemblies in biological membranes and in model membrane systems. This experimental approach requires that the hybrid species in the membrane have a distinctive property that is readily detected. Single-channel conductance and fluorescence characteristics are especially well-suited for such studies because they can be measured sensitively and precisely. In particular, fluorescence energy transfer is a useful indicator of a hybrid species, provided that the donor and acceptor chromophores are within about 40 Å of each other. The relative fluorescence yield of the donor as a function of the mole fraction of the acceptor can then be used to distinguish amongst various oligomeric models. Single-channel conductance measurements are ideal for the detection of hybrid channels, provided that the pure species have sufficiently different conductance values. This may be achieved by chemical modification, as in this study. Alternatively, it may be feasible to form distinctive hybrid channels in reconstituted membranes by mixing subunits of related, naturally-occurring molecules, such as acetylcholine receptors from different species.

What is the Conformation of the Gramicidin Channel Dimer?

Now that the dimeric nature of the channel is established, we can turn to these questions: What is the detailed conformation of the channel? How does it interact with ions and with membrane phospholipids? We know from the simultaneous conductance and fluorescence measurements that nearly all gramicidin dimers are channels, and we know from the fluorescence energy transfer measurements that nearly all of the gramicidin in liposomes is dimerized. Hence, spectroscopic studies of gramicidin in liposomes should provide information about the structure and dynamics of the transmembrane channel. It should be feasible to determine whether the conformation of the channel corresponds to the π_6 (L, D) helical dimer (Fig. 16a) proposed by Urry (21, 22), or to one of the parallel or anti-parallel-β double helices (Fig. 16b) proposed by Veatch et al. (23). All of these models provide a polar channel about 4 Å in diameter and about 25–30 Å in length. All have hydrophobic exteriors to interact favorably with the membrane interior and have more polar ends to stabilize the channel in the transmembrane configuration.

Urry et al. (22) have reported the preparation of a covalent gramicidin dimer in which the formyl group of each gramicidin has been removed and the 2 amino groups joined by a malonic acid linkage. This is equivalent to replacing the 2 formyl protons by a methylene bridge. This material does appear to have many of the membrane conductance characteristics expected for a unimolecular channel. However, I have used silica thin layer chromatography to fractionate samples of this material (kindly supplied by Dr. Dan Urry), and the membrane activity does not appear to comigrate with the major chemical component. Hence, there is some doubt as to the chemical identity of the membrane active species.

If the formyl groups are indeed very close together in the channel dimer, then the anti-parallel-β double helix (Fig. 16b) is ruled out. Both the parallel-β double helix (not shown) and the π_6 (L, D) helical dimer (Fig. 16a) place the formyl groups in close proximity. Further measurements will be required to distinguish between these possibilities.

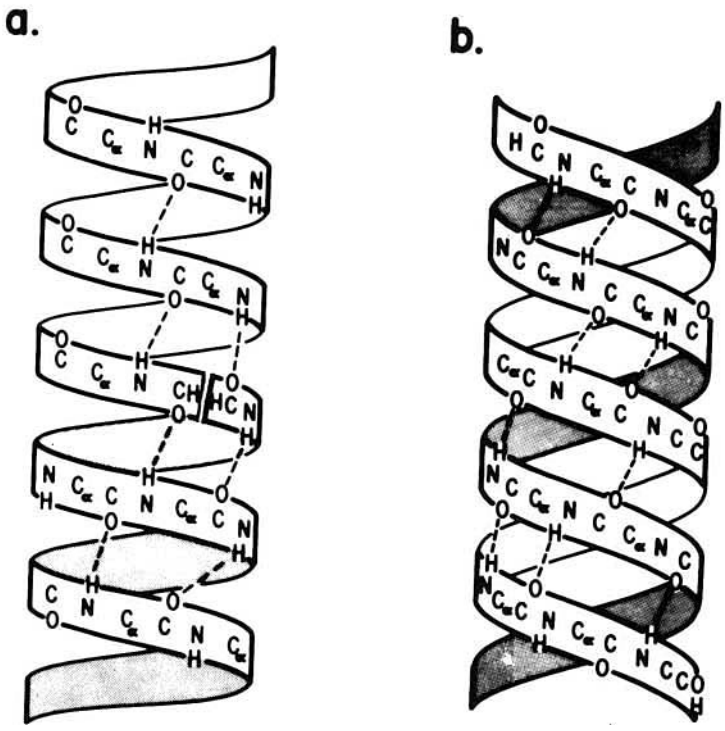

Fig. 16. Proposed models of the conformation of the gramicidin dimer channel. These schematic diagrams show the conformation of the polypeptide backbone with the side chains removed. Hydrogen bonds are shown as dashed lines and 1 chain is shown shaded. (a) Urry's π_6 (L, D) helical dimer. (b) The antiparallel-β double helix proposed by Veatch et al. (23). It is also possible to form a parallel-β double helix (not shown) in which the chains run in the same direction.

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