EQUILIBRIUM BINDING CONSTANTS FOR Tl^+ WITH GRAMICIDINS A, B AND C IN A LYSOPHOSPHATIDYLCHOLINE ENVIRONMENT DETERMINED BY ^{205}Tl NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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ABSTRACT Nuclear Magnetic Resonance (NMR) ^{205}Tl spectroscopy has been used to monitor the binding of Tl^+ to gramicidins A, B, and C packaged in aqueous dispersions of lysophosphatidylcholine. For 5 mM gramicidin dimer in the presence of 100 mM lysophosphatidylcholine, only ~50% or less of the gramicidin appears to be accessible to Tl^+. Analysis of the ^{205}Tl chemical shift as a function of Tl^+ concentration over the 0.65–50 mM range indicates that only one Tl^+ ion can be bound by gramicidin A, B, or C under these experimental conditions. In this system, the Tl^+ equilibrium binding constant is 582 ± 20 M^{-1} for gramicidin A, 1949 ± 100 M^{-1} for gramicidin B, and 390 ± 20 M^{-1} for gramicidin C. Gramicidin B not only binds Tl^+ more strongly but it is also in a different conformational state than that of A and C, as shown by Circular Dichroism spectroscopy. The ^{205}Tl NMR technique can now be extended to determinations of binding constants of other cations to gramicidin by competition studies using a ^{205}Tl probe.

INTRODUCTION

The gramicidin family of linear polypeptides represents a set of well-defined transmembrane ion channels (Andersen, 1984). These channels are ideally selective for monovalent cations and partially selective among the different monovalent cations (Myers and Haydon, 1972; Eisenman et al., 1976; Urban et al., 1980), which move through the channels in single file (Finkelstein and Andersen, 1981). The intimate chemical interactions between the passing ions and the gramicidin channel can be probed through studies of single-site analogues in which individual amino acids of the parent gramicidin A molecule are substituted (Mazet et al., 1984). Thus the single-file transport of ions in the gramicidin channel system can be investigated at the molecular level in greater detail than is currently feasible for any other transmembrane channel.

The parent gramicidin A molecule from *Bacillus brevis* has the amino acid sequence (Sarges and Witkop, 1965*a*)

formyl—L—Val—Gly—L—Ala—D—Leu—L—
(1) (2) (3) (4)

Ala—D—Val—L—Val—D—Val—L—
(5) (6) (7) (8)

Trp—D—Leu—L—Trp—D—Leu—L—
(9) (10) (11) (12)

Trp—D—Leu—L—Trp—ethanolamine.
(13) (14) (15)

Analogues having single amino acid substitutions are available either from *B. brevis* (Gregory and Craig, 1948) or by total chemical synthesis (Prasad et al., 1982) or semisynthesis (Morrow et al., 1979; Weiss and Koeppe, 1985). This paper will focus on the three naturally occurring gramicidins produced by *B. brevis* that differ only in the amino acid at position 11: gramicidins A, B, and C, having *Trp*, *Phe*, and *Tyr*, respectively, at position 11 (Sarges and Witkop, 1965*b*). The carbonyl carbon of residue 11 is near the tight cation binding site of gramicidin, as determined by C-13 Nuclear Magnetic Resonance (NMR) spectroscopy (Urry et al., 1982). The formyl-to-formyl single-stranded $\beta^{6.3}$-helical dimer (Urry, 1971) has now been firmly established by spectroscopic and

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chemically labeling techniques (Urry et al., 1971; Bamberg et al., 1977; Weinstein et al., 1979; Urry et al., 1983) as the structure of the ion-transporting gramicidin channel. In this model, the side chains of gramicidin do not directly contact the passing ions; an aromatic side chain at position 11 extends over an area about 5–10 Å away from a cation coordinated to the carbonyl of residue 11. Nevertheless, individual side chains can dramatically influence the conductance properties of gramicidin channels.

When the single-channel conductances of gramicidins A, B, and C are compared in phosphatidylcholine or glycerol monooleate membranes at a variety of permeant ion activities (Bamberg et al., 1976), gramicidins A and C exhibit similar conductances, whereas the conductance of gramicidin B channels is consistently about a third lower than gramicidin A or C. This lower conductance when Phe is present at position 11 remains a challenge for theoretical treatments (Jordan, 1982) and molecular dynamics simulations (MacKay et al., 1984) of ion permeation through gramicidin channels. To obtain more insight about the reason for the lower conductance of gramicidin B, and to place constraints on possible kinetic models for single-file transport, we have sought to measure accurately equilibrium cation binding constants for gramicidins A, B, and C.

A number of factors indicate that the thallous ion (Tl^+) is a convenient and relevant ion for studies of equilibrium binding to gramicidins: (*a*) Tl^+ is transported through the channel. Its ionic radius is intermediate in size between Na^+ and Cs^+, which are also transported. (*b*) Tl^+ is more strongly bound to gramicidins than are the alkali cations. Low concentrations of Tl^+ inhibit transport of K^+ or Na^+ (Neher, 1975; Andersen, 1975). The tighter binding of Tl^+ means that binding constants can be determined with greater precision, and differences among gramicidin analogues can be measured with greater confidence. The overall binding constant for Tl^+ to a naturally occuring mixture of gramicidins A, B, and C has been shown to be 500–1000 M^{-1} (Veatch and Durkin, 1980; Hinton et al., 1982). (*c*) ^{205}Tl NMR spectroscopy provides an extraordinarily sensitive probe of the environment of Tl^+, since the observed chemical shift varies over a range of 1,000 ppm (Briggs et al., 1980). Thus, the equilibrium between free and gramicidin-bound Tl^+ can be measured with high precision.

The conformation of gramicidin is known to be quite sensitive to environment (Urry, 1973; Urry et al., 1975; Wallace et al., 1981). We therefore packaged the gramicidins in phospholipid structures, dispersed in aqueous solutions, before the Tl-binding measurements. The lysophosphatidylcholine system described by Urry and coworkers (Spisni et al., 1979) is convenient because relatively high gramicidin concentrations can be achieved for good NMR sensitivity. Circular dichroism (CD) spectroscopy indicates that the conformation of gramicidin A in L-α-Lysophosphatidylcholine (lyso-PC) dispersions is likely to be quite similar to that in bilayer membranes and vesicles (Urry et al., 1979). Gramicidin B, however, has a different conformation to that of A (Prasad et al., 1983). This paper reports the results of ^{205}Tl NMR investigations of the Tl^+ binding constants for gramicidins A, B, and C in lyso-PC environments.

MATERIALS AND METHODS

Materials

Mixtures of gramicidins A, B, and C were purchased as "gramicidin D" from Sigma Chemical Company (St. Louis, MO) or as "gramicidin" from U.S. Biochemical Corporation (Cleveland, OH). The individual A, B, and C components were separated on a 0.78 × 240 cm column of Chromosorb LC-5, a phenyl reversed phase 37–44 μm pellicular packing (Johns–Manville, Denver, CO), using a multi-step 70-h water-methanol gradient, as previously described (Koeppe et al., 1985). The gramicidins were further purified on Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ). Gramicidin concentrations were determined by vacuum drying to constant weight, and verified by UV spectroscopy (ϵ_{280} = 20,840 M^{-1}, 15,260 M^{-1}, and 18,600 M^{-1} for gramicidins A, B, and C, respectively). The relative amounts of the A, B, and C components were assayed by analytical HPLC (Koeppe and Weiss, 1981). The three samples used for NMR spectroscopy consisted of (81% C, 19% A), (92% A, 8% C) and (87% B, 13% A).

Lyso-PC from egg yolk, containing primarily palmitic and stearic acids at position one, was obtained from Sigma and was used without further purification. Thallous nitrate from Alfa Products (Danvers, MA) was recrystallized twice from H_2O and dried to constant weight at 90°C, 40 mm Hg. Water was deionized and then glass distilled. All other chemicals were reagent grade.

NMR Samples and Measurements

Gramicidin was incorporated into lyso-PC micelles using the procedure of Urry (Urry et al., 1979; Spisni et al., 1979; Hinton et al., 1982). The samples prepared in this manner from very pure gramicidins produced solutions that were remarkably clear, unlike those obtained from the commercial mixture of gramicidins. Samples for NMR spectroscopy in 5 mm tubes were prepared by adding 0.1 ml of degassed aqueous $TlNO_3$ to 0.4 ml of a lyso-PC plus gramicidin suspension, to yield 5 mM gramicidin dimer, 100 mM lyso-PC, and the desired Tl^+ concentration. For Tl^+ concentrations below 6 mM, samples of 1.5 ml total volume in 10 mm NMR tubes were used. Following the recording of initial spectra, additional samples having intermediate Tl^+ concentrations were prepared by pairwise mixing of previous samples. All samples in NMR tubes were maintained at 68°C for the duration of a set of experiments.

Aliquots of selected samples were diluted 100-fold with distilled water for the CD measurements over the 200–300 nm range using a spectrometer (model J-500A; Jasco Inc., Easton, MD).

The ^{205}Tl NMR spectra were recorded at 34°C, using a spectrometer modified for pulsed-FT operation (model HFX-90; Bruker Instruments Inc., Billerica, MA) with a temperature-controlled probe. The gramicidin induced shift of the $^{205}Tl^+$ ion resonance frequency was determined as a function of the Tl^+ ion concentration at constant gramicidin A, B, or C concentration. Under the conditions of the experiments, the Tl^+ ions were in rapid exchange between "free" and gramicidin-bound states and only one resonance signal was observed for each sample. To correct for the lyso-PC-induced shift in the $^{205}Tl^+$ resonance frequency, the shift produced by lipid samples devoid of any gramicidin was subtracted from the shift due to the gramicidin-containing phospholipid. All chemical shifts are referenced to that of the Tl^+ ion in water at infinite dilution with downfield shifts assigned positive values.

Data Analysis

One can express the interaction of the Tl^+ ion with the gramicidin dimer (Gr_2) incorporated into micelles by the following equilibria:

$$Tl^+ + Gr_2 \rightleftharpoons Tl^+Gr_2 \quad (1)$$

$$K_1 = [Tl^+Gr_2]/[Tl^+]\,[Gr_2] \quad (2)$$

and

$$Tl^+ + Tl^+Gr_2 \rightleftharpoons Tl_2^{++}Gr_2 \quad (3)$$

$$K_2 = [Tl_2^{++}Gr_2]/[Tl^+]\,[Tl^+Gr_2]. \quad (4)$$

For Tl^+ ions rapidly exchanging between "free" and bound states, the observed chemical shift, δ_{obs}, is given by

$$\delta_{obs} = \chi_f\delta_f + \chi_1\delta_1 + \chi_2\delta_2, \quad (5)$$

where χ_f, χ_1, and χ_2 are the mole fractions and δ_f, δ_1, and δ_2 are the chemical shifts of the free, single and doubly bound complexes, respectively.

One must extract from the experimentally determined $^{205}Tl^+$ chemical shifts, the equilibrium binding constants (K_1 and K_2) and the bound chemical shifts (δ_1 and δ_2). The procedure followed for obtaining these parameters was that of Johnston et al. (1975).

Since no gramicidin sample was entirely pure, the experimental chemical shift data for each sample were corrected for the shift produced by the minor gramicidin component. The highest purity sample, gramicidin A (92% A and 8% C), was used as the basis for correcting all of the samples in the following manner. The analysis of the chemical shift data of the gramicidin A sample gave values for K_1 and δ_1. Assuming these values to be approximately equal to those for pure gramicidin A, the chemical shift contribution of gramicidin A to the gramicidin C sample (81% C and 19% A) was calculated point by point and subtracted from the observed chemical shift data for the gramicidin C sample. The K_1 and δ_1 parameters were then obtained for the corrected gramicidin C sample. These values for the gramicidin C sample were then used to correct the gramicidin A data and to calculate new values of K and δ_1 for gramicidin A. The new values for gramicidin A were then used again to correct the gramicidin C data. This iterative correction procedure was continued until constant values were obtained that represent K_1 and δ_1 for pure gramicidins A and C. The final values for pure gramicidin A were then used to correct the data for the gramicidin B sample (87% B and 13% A).

The accurate determination of binding constant values from experimental curves relating chemical shift and metal ion concentration is a difficult task. Deranleau (1969) has shown that ~75% of the saturation curve is required to show the correspondence between the equation of the model and the equation fitting the data. The concentration of the metal ion (Tl^+) must be corrected for ionic strength effects. The extended Debye-Huckel equation (Bockris and Reddy, 1970) was used to determine the activity coefficient of Tl^+. One must be concerned about the effective concentration of gramicidin dimer that is accessible to Tl^+ in these lipid systems. These points will be illustrated using the data for gramicidin A (uncorrected for the 13% of gramicidin C that it contained).

RESULTS

The lipid correction for Tl^+ binding to lyso-PC in the absence of a gramicidin is very small, even at low ionic strength (Fig. 1). Thus for $TlNO_3$ and lyso-PC, one need not be concerned about specific adsorption of Tl^+ to the lipid surface at low ionic strength. Such an effect could have increased the local cation concentration in the vicinity of the channel mouth (Andersen, 1983).

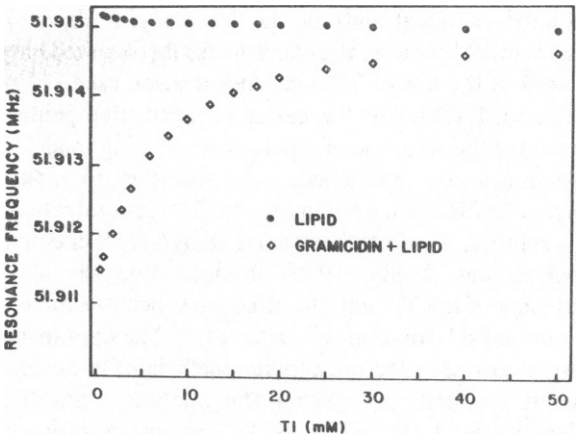

FIGURE 1 Lipid correction curve with the associated chemical shift data for gramicidin A for comparison.

Table I contains the best fit values of K_1 as a function of the lowest Tl^+ ion concentration used in the analysis. The fits are characterized by the Q factor, which measures the "best-fit" to the experimental data [$Q = \Sigma_{n-1}^{n}(\delta_{obs} - \delta_{cal})^2$]. For all of these fits, the gramicidin concentration was the analytical value of 5 mM dimer. As one can see from this table, the experimental data can apparently be fit very well when the lowest Tl^+ ion concentration is ~6 mM. However, as one includes lower Tl^+ ion concentration data, the fit becomes precipitously worse (i.e., the value of Q gets very large). This illustrates the problem of not covering a large enough portion of the saturation curve (i.e., not using low enough Tl^+ ion concentrations). This problem is so serious that one can appear to get a very good fit (Q = 0.22) to the data for $Tl^+ \geq 8$ mM using an erroneous two K model with the parameters $K_1 = 1{,}317\ M^{-1}$, $K_2 = 5.7\ M^{-1}$, $\delta_1 = 57.3$ ppm and $\delta_2 = 7.9$ ppm. The key to being able to rule out this fit to a two K model is the fact that the

TABLE I
APPARENT BINDING CONSTANT, K_1 (M^{-1}), AND Q AS A FUNCTION OF THE LOWEST Tl^+ CONCENTRATION USED IN THE CALCULATION*

Tl^+	K_1	Q
mM	M^{-1}	
8	932	0.271
6	980	0.307
4	1442	5.03
3	1980	20.4
2	2100	72
1	1700	190
0.65	1449	327

*The analytical gramicidin A dimer concentration of 5 mM was used in these calculations. No activity coefficient correction was made for the Tl^+ ion in obtaining these values.

predicted chemical shift of the 1:1 complex (δ_1 = 57.3 ppm) is much less than the experimentally observed chemical shift at the lowest Tl^+ ion concentration used (71 ppm at 0.65 mM Tl^+). The low cation concentration points are crucial for the selection of a particular binding model.

As previously mentioned, it is necessary to make an activity coefficient correction to the Tl^+ concentration for each solution. Using the extended Debye-Huckel equation (Bockris and Reddy, 1970) to determine the activity coefficient of the Tl^+ ion, the effective concentration of the Tl^+ ion in each solution was calculated. The data in Table II were corrected for the activity coefficient; however, the best fit was still poor using the analytical gramicidin concentration. Using all of the Tl^+ ion concentration data (i.e., including the lowest analytical value of 0.65 mM), the two-site model produced a better fit (Q = 24.9) than did the one-site model (Q = 505), but with $K_2 > K_1$. The only satisfactory fits to the data were found when the analytical gramicidin dimer concentration was reduced to a minimized effective concentration for the calculation. Table II contains the data for gramicidin A that illustrates this point with the one K model using Tl^+ ion concentrations corrected by activity coefficients. As can be clearly seen, the Q value minimizes as the effective concentration is lowered to about half of the analytical dimer concentration of 5 mM (actually a value of 2.4 mM was obtained for the value of gramicidin A when correction was made for the 13% of gramicidin C in the sample). For gramicidins B and C, the effective concentrations of the dimer that gave the best fits to the data were 1.5 and 2.7 mM, respectively. An experiment was also performed with gramicidin A at an analytical concentration of 3 mM. A best fit analysis of these data was obtained with an effective dimer concentration of 1.47 mM, again about half of the actual concentration. This fit gave values of K_1 and δ_1 that were in very good agreement with those obtained with the system using an analytical dimer concentration of 5 mM. Thus, four separate experiments have all indicated that the effective gramicidin concentration must be lowered to fit the data.

The process of varying the effective dimer concentration proved to be crucial in fitting the data for the ^{205}Tl chemical shift in the presence of all of the gramicidins. In all experiments, a minimum was observed in the dependence of Q on effective gramicidin concentration. An example of this is shown in Fig. 2 for gramicidin C.

The theoretical curves that best fit each of the gramicidins are shown in Fig. 3. The curves in Fig. 3 are all three-parameter fits in which K_1, δ_1, and the effective gramicidin concentration were optimized. The effective gramicidin concentration was in each case ~50% of the analytical concentration for A and C and somewhat lower for B. Attempts were made to include a second binding site with characteristic K_2 and δ_2 parameters in each of the fits. In all of the two-site fits K_2 tended toward zero ($<10\ M^{-1}$) and δ_2 assumed unrealistic negative values. Therefore, the possibility of two simultaneously bound Tl^+ ions per gramicidin dimer was excluded for the experimental conditions described here. The Tl^+ binding constants, K_1, obtained by the "best-fit" procedure are 582 ± 20, 1949 ± 100, and 390 ± 20 M^{-1} for gramicidins A, B, and C, respectively, at 34°C. The associated bound chemical shifts, δ_1, are 127 ± 2, 43 ± 2, and 93 ± 2 for gramicidins, A, B, and C, respectively.

TABLE II

BINDING CONSTANT, K (M^{-1}), Q, AND δ (PARTS PER MILLION) CALCULATED AS A FUNCTION OF EFFECTIVE GRAMICIDIN DIMER CONCENTRATION WITH Tl^+ CORRECTED FOR ACTIVITY COEFFICIENT

Gramicidin A dimer (mM)	5.0	3.0	2.6	2.5	2.4	2.0
K_1 (0.65 mM) M^{-1}	1802	1164	846	767	721	532
Q (0.65 mM)	505	16.7	3.96	1.88	2.15	6.36
δ (0.65 mM) ppm	65.8	91.6	108	113	119	150
K_1 (0.65 mM) M^{-1}	496			696*		
K_2 (0.65 mM) M^{-1}	837			2.58*		
Q (0.65 mM)	24.9			2.26*		
δ_1 (0.65 mM) ppm	118			118*		
δ_2 (0.65 mM) ppm	19.9			−8.62*		

*This calculation was terminated before it reached the best fit values to show the direction that it was proceeding. If the calculation is allowed to go to completion, K_2 goes to zero and δ_2 becomes an extremely large negative number.

DISCUSSION

Thallium exhibits unusual electrophysiological and magnetic resonance properties which render the Tl^+ cation a remarkable probe for mechanistic studies of Na^+ and K^+ gates and pumps in biological membranes. ^{205}Tl is a convenient nucleus for NMR because of its high natural abundance (70.5%), its excellent relative receptivity (0.136 relative to ^1H), and its lack of a quadrupole moment.

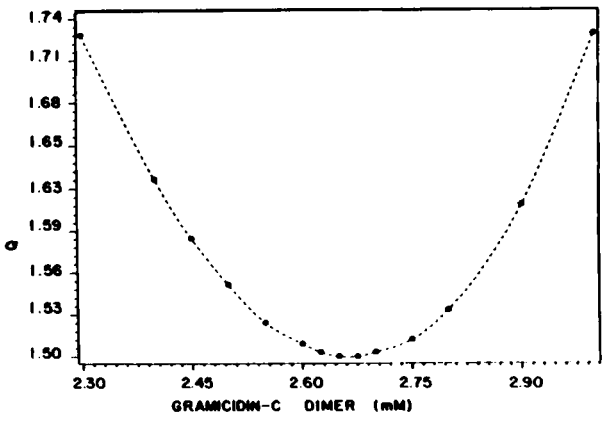

FIGURE 2 Q factor as a function of effective gramicidin C concentration. The minimum Q occurs at 2.7 mM gramicidin C dimer, or 54% of the analytical concentration.

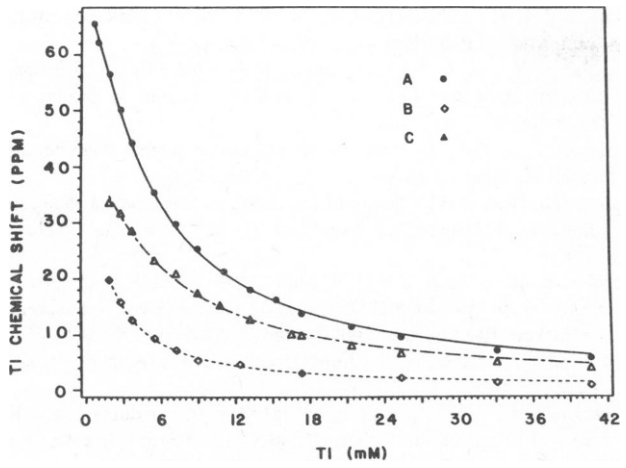

FIGURE 3 Theoretical curve fits to the ^{205}Tl chemical shift data for gramicidins A, B, and C.

Furthermore, the ^{205}Tl chemical shift is extremely sensitive to the chemical environment.

Tl^+ substitutes with high affinity at the K^+ binding sites of the $Na^+ = K^+$ ATPase of human erythrocytes (Cavieres and Ellory, 1974), and Tl^+ is actively transported into erythrocytes in exchange for internal Na^+. Tl^+ is also the most permeable ion in the K^+ channel of squid (Hagiwara et al., 1972) and binds more strongly than Na^+ or K^+ to the Na^+ channel of nerve membrane (Henderson et al., 1974). In the case of gramicidin, 10^{-2} M Tl^+ is 50 times more permeable than 10^{-2} M K^+ (Eisenman et al., 1976). However, Na^+ or K^+ currents through gramicidin channels are depressed by traces of Tl^+. In symmetric mixtures of Na^+ and Tl^+, the conductance depends on the ratio of $[Tl^+]/[Na^+]$ and for 1 M total salt passes through a minimum at ~2% thallium (Neher, 1975; Andersen, 1975). This "mole-fraction-dependent" behavior provides an example of complex interactions among different cations and gramicidin channels (Andersen, 1978).

The fact that Tl^+ binds more strongly than Na^+ or K^+ to gramicidins can be exploited to obtain accurate cation association constant comparisons among closely related gramicidins. Thus Tl^+ is a useful probe for determining the relationships between ion affinities and single-channel conductances for the gramicidin family. Of course, the detailed transport mechanism may be different for different cations, and so further single-channel studies with Tl^+ and equilibrium studies with Na^+ and K^+ will be required.

The results of the "best-fit" analysis show that only one Tl^+ ion is bound by gramicidin A, B, or C incorporated into lyso-PC. Furthermore, a comparison of the equilibrium binding constants indicates that the Tl^+ binding constant for gramicidins A and C are similar, whereas that of B is larger by a factor of about three. It is of interest to note that the order of increasing equilibrium binding constants, gramicidin C < gramicidin A < gramicidin B is the same as that for the retention times, and therefore the polarity, of the gramicidins found in reversed phase chromatography (Koeppe and Weiss, 1981). The difference in equilibrium binding constants among the A, C, and B components suggests that the energy profile at the channel entrance is significantly altered for gramicidin B in the lyso-PC environment. The CD spectra of gramicidins A, B, and C incorporated into the lipid environment show that A and C are in a very similar conformational state with that of B being different. Our CD results for A and B are in agreement with those of Prasad et al. (1983) for synthetic gramicidin B. Since the conformational and Tl^+ binding properties of B are different than those for A or C in the lyso-PC environment, one might expect to observe significant differences in the single channel conductances of these gramicidin analogs. Single-channel conductance studies of the Na^+ and K^+ ions with gramicidins A, B, and C have shown that the conductance of gramicidin B is ~60–70% of that of gramicidin A, while A and C have about the same conductance at NaCl concentrations of 0.1–1.0 M (Bamberg et al., 1976). The single channel conductance of Na^+ (1 M, 200 mV, 25°C in diphytanoyl-PC) through gramicidins A, B, and C was found to be 15.4, 8.2, and 15.5 pS, respectively (Mazet et al., 1984). Unless one is certain that the channel state is the same in the conductance and equilibrium binding experiments, correlations between transport and binding must be cautiously made.

The magnitude of the equilibrium binding constant determined for gramicidin-A (582 M^{-1}) is in agreement with the value range of 500–1,000 M^{-1} determined by an equilibrium dialysis method at 23°C with gramicidin A incorporated into vesicles of dimyristoyl-PC (Veatch and Durkin, 1980). The equilibrium dialysis experiment also showed only one Tl^+ ion bound by the gramicidin dimer, in agreement with our results. Levitt (1978) predicted that only one Tl^+ ion would be bound by gramicidin with a binding affinity of 400–500 M^{-1}. Previously, we measured an equilibrium binding constant for the Tl^+ ion with a commercial mixture of gramicidins incorporated into micelles using the ^{205}Tl NMR technique (Hinton et al., 1982). A one-binding-site model was used to fit the data and a value of 900 M^{-1} was obtained for the equilibrium binding constant at 30°C. The gramicidin mixture used in the earlier experiments was composed of ~85% A, 10% B, and 5% C. The results presented here for gramicidin A are consistent with those determined previously for the gramicidin mixture, and the precision of the analysis is now much improved.

The importance of using the effective concentration and not the analytical gramicidin concentration for the data analysis is obvious. However, the factors that determine the effective concentration in lipid dispersions are not well understood. It has been shown, however, that this type of lipid system can form complex mixtures of bilayers upon the addition of gramicidin (Spisni et al., 1983; Killian et al., 1983). Perhaps it is within the bilayers that some of the

gramicidin becomes inaccessible. Several different gramicidin A experiments showed the effective concentration ranged from ~2.4 to 3.0 mM with an analytical concentration of 5 and 1.47 mM with an analytical concentration of 3 mM. The binding constant remained the same in each case. Slight variations in the phospholipid and in the incorporation of gramicidin are probably responsible for the differences observed in the effective concentrations. It is not our belief that the effective concentration differences reflect upon intrinsic differences in molecular properties of the gramicidins. The data indicate that, for the concentrations of gramicidin and lyso-PC that were used, only about half of the total gramicidin and only one end of each dimer is available for cation binding at any one time. The process by which only part of the gramicidin becomes available for cation binding would seem to be a dynamic one since Urry et al. (1982) have observed only one ^{13}C-carbonyl resonance signal for singly labeled, ^{13}C-enriched gramicidin incorporated into lyso-PC both in the presence and absence of cation binding. These ^{13}C-NMR experiments indicate that the two ends of a gramicidin dimer are equivalent on the NMR time scale. This dynamic behavior is supported by the dielectric relaxation studies of Henze et al. (1982), who found intrachannel ionic translocation from the site at one end to that on the other proceeds with a rate constant of 4×10^6 s^{-1}. Taken together, the ^{205}Tl and ^{13}C data indicate that a single bound Tl^+ ion is in dynamic equilibrium between the two ends of the channel.

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REFERENCES

Andersen, O. S. 1975. Ion-specificity of gramicidin A channels. Fiftieth International Biophysics Congress, Copenhagen. 112. (Abstr.)

Andersen, O. S. 1978. Ion transport across simple membranes. *In* Renal Function. G. H. Giebisch and E. F. Purcell, editors. Josiah Macy, Jr. Foundation. New York. 71–99.

Andersen, O. S. 1983. Ion movement through gramicidin A channels. Studies on the diffusion-controlled association step. *Biophys. J.* 41:147–165.

Andersen, O. S. 1984. Gramicidin Channels. *Annu. Rev. Physiol.* 46:531–548.

Bamberg, E., H.-J. Apell, and H. Alpes. 1977. Structure of the gramicidin A channel: discrimination between the $\tau_{L,D}$ and the β-helix by electrical measurements with lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA.* 74:2402–2406.

Bamberg, E., K. Noda, E. Gross, and P. Lauger. 1976. Single-channel parameters of gramicidin A, B and C. *Biochim. Biophys. Acta.* 419:223–228.

Bockris, J. O'M., and Reddy, A. K. N. 1970. Modern Electrochemistry. Vol. 1. Plenum Publishing Corp., New York. 175–286.

Briggs, R. W., F. A. Etzkorn, and J. F. Hinton. 1980. ^{205}Tl nuclear magnetic resonance study of the thallium complex of lasalocid. *J. Magn. Reson.* 37:523–528.

Cavieres, J. D., and J. C. Ellory. 1974. Thallium and the sodium pump in human red cells. *J. Physiol.* (Lond.). 243:243–266.

Deranleau, D. A. 1969. Theory of the measurement of weak molecular complexes. I. General considerations. *J. Am. Chem. Soc.* 91:4044–4049.

Eisenman, G., S. Krasne, and S. Ciani. 1976. Further studies on ion selectivity. *In* International Workshop on Ion Selective Electrodes and on Enzyme Electrodes in Biology and in Medicine. Kessler, Clark, Lubbers, Silver and Simon, editors. Urban & Schwarzenberg, Munich. 3–21.

Finkelstein, A., and O. S. Andersen. 1981. The gramicidin A channel: a review of its permeability characteristics with special reference to the single-file aspect of transport. *J. Membr. Biol.* 59:155–171.

Gregory, J. D., and L. C. Craig. 1948. Counter-current distribution of gramicidin. *J. Biol. Chem.* 172:839–840.

Hagiwara, S., D. C. Eaton, A. E. Stuart, and N. P. Rosenthal. 1972. Cation selectivity of the resting squid axon membrane. *J. Membr. Biol.* 9:373–384.

Henderson, R., J. M. Ritchie, and G. R. Strichartz. 1974. Evidence that tetrodotoxin and saxitoxin act at a metal cation binding site in the sodium channels of nerve membrane. *Proc. Natl. Acad. Sci. USA.* 71:3936–3940.

Henze, R., E. Neher, T. L. Trapane, and D. W. Urry. 1982. Dielectic relaxation studies of ionic processes in lysolecthin-packaged gramicidin channels. *J. Membr. Biol.* 64:233–239.

Hinton, J. F., G. Young, and F. S. Millett. 1982. Thallous ion interaction with gramicidin incorporated in micelles studied by thallium-205 nuclear magnetic resonance. *Biochemistry.* 21:651–654.

Johnston, M. D., B. L. Shapiro, M. J. Shapiro, T. W. Proulx, A. D. Godwin, and H. L. Pearce. 1975. Lanthanide-induced shifts in proton NMR spectra. XI. Equilibrium constants and bound shifts for cyclohexanones and cyclohexanols. *J. Am. Chem. Soc.* 97:542–554.

Jordan, P. C. 1982. Electrostatic modeling of ion pores. Energy barriers and electric field profiles. *Biophys. J.* 39:157–164.

Killian, J. A., B. DeKruijff, C. J. A. van Echteld, A. J. Verkleij, J. Leunissen-Bijvelt, and J. DeGier. 1983. Mixtures of gramicidin and lysophosphatidylcholine form Lamellar structures. *Biochim. Biophys. Acta.* 728:141–144.

Koeppe, R. E. II, and L. B. Weiss. 1981. Resolution of linear gramicidins by preparative reversed-phase high-performance liquid chromatography. *J. Chromatogr.* 208:414–418.

Koeppe, R. E. II, J. A. Packowski, and W. L. Whaley. 1985. Gramicidin K, a new linear channel-forming gramicidin from *Bacillus brevis*. *Biochemistry.* 24:2822–2826.

Levitt, D. G. 1978. Electrostatic calculations for an ion channel. *Biophys. J.* 22:209–248.

MacKay, D. H. J., P. H. Berens, K. R. Wilson, and A. T. Hagler. 1984. Structure and dynamics of ion transport through gramicidin A. *Biophys. J.* 46:229–248.

Mazet, J.-L., O. S. Andersen, and R. E. Koeppe II. 1984. Single-channel studies on linear gramicidins with altered amino acid sequences: A comparison of phenylalanine, tryptophan, and tyrosine substitution at positions 1 and 11. *Biophys. J.* 45:263–276.

Morrow, J. S., W. R. Veatch, and L. Stryer. 1979. Transmembrane channel activity of gramicidin A analogs: Effects of modification and deletion of the amino-terminal residue. *J. Mol. Biol.* 132:733–738.

Myers, V. B., and D. A. Haydon. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. II. The ion selectivity. *Biochim. Biophys. Acta.* 274:313–322.

Neher, E. 1975. Ionic specificity of the gramicidin channel and the thallous ion. *Biochim. Biophys. Acta.* 401:540–544. Errata ibid. 1977. 469:359.

Prasad, K. U., T. L. Trapane, D. Busath, G. Szabo, and D. W. Urry. 1982. Synthesis and characterization of 1-^{13}C-D-Leu^{12,14} gramicidin A. *Int. J. Peptide Protein Res.* 19:152–171.

Prasad, K. U., T. L. Trapane, D. Busath, G. Szabo, and D. W. Urry. 1983. Synthesis and characterization of (1-^{13}C)Phe gramicidin A. *Int. J. Peptide Protein Res.* 22:341–347.

Sarges, R., and B. Witkop. 1965*a*. Gramicidin V. The structure of valine- and isoleucine-gramicidin A. *J. Am. Chem. Soc.* 87:2011–2020.

Sarges, R., and B. Witkop. 1965*b*. Gramicidin VIII. The structure of valine- and isoleucine-gramicidin C. *Biochemistry.* 4:2491–2494.

Spisni, A., M. A. Khaled, and D. W. Urry. 1979. Temperature-induced incorporation of gramicidin A into lysolecithin micelles demonstrated by ^{13}C NMR. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 102:321–324.

Spisni, A., I. Pasquali-Ronchetti, E. Casali, L. Lindner, P. Cavatorta, L. Masotti, and D. W. Urry. 1983. Supramolecular organization of Lyso-phosphatidylcholine-packaged gramicidin A. *Biochim. Biophys. Acta.* 732:58–68.

Urban, B. W., S. B. Hladky, and D. A. Haydon. 1980. Ion movements in gramicidin pores. An example of single-file transport. *Biochim. Biophys. Acta.* 602:331–354.

Urry, D. W. 1971. The gramicidin A transmembrane channel: a proposed π(L, D) Helix. *Proc. Natl. Acad. Sci. USA.* 68:672–676.

Urry, D. W. 1973. Polypeptide conformation and biological function of β-helices as permselective transmembrane channels. *In* Conformation of Biological Molecules and Polymers. The Jerusalem Symposia on Quantum Chemistry and Biochemistry. Vol. 5. E. D. Bergmann and B. Pullman, editors. Israel Academy of Sciences. Jerusalem. 723–736.

Urry, D. W., M. C. Goodall, J. D. Glickson, and D. F. Mayers. 1971. The gramicidin A transmembrane channel: Characteristics of head-to-tail dimerized $\pi_{L,D}$ helices. *Proc. Natl. Acad. Sci. USA.* 68:1907–1911.

Urry, D. W., M. M. Long, M. Jacobs, and R. D. Harris. 1975. Conformation and molecular mechanisms of carriers and channels. *Ann. N.Y. Acad. Sci.* 264:203–220.

Urry, D. W., K. U. Prasad, and T. L. Trapane. 1982. Location of monovalent cation binding sites in the gramicidin channel. *Proc. Natl. Acad. Sci. USA.* 79:390–394.

Urry, D. W., A. Spisni, and M. A. Khaled. 1979. Characterization of micellar-packaged gramicidin A channels. *Biochem. Biophys. Res. Commun.* 88:940–949.

Urry, D. W., T. L. Trapane, and K. U. Prasad. 1983. Is the gramicidin A transmembrane channel single-stranded or double-stranded helix? A simple unequivocal determination. *Science. (Wash. DC).* 221:1064–1067.

Veatch, W. R., and J. T. Durkin. 1980. Binding of thallium and other cations to the gramicidin A channel: Equilibrium dialysis study of gramicidin in phosphatidylcholine vesicles. *J. Mol. Biol.* 143:411–417.

Wallace, B. A., W. R. Veatch, and E. Blout. 1981. Conformation of gramicidin A in phospholipid vesicles: Circular dichroism studies of the effects of ion binding, chemical modification and lipid structure. *Biochemistry.* 20:5754–5760.

Weinstein, S., B. A. Wallace, E. R. Blout, J. S. Morrow, and W. R. Veatch. 1979. Conformation of gramicidin A in phospholipid vesicles: A ^{13}C and ^{19}F NMR study. *Proc. Natl. Acad. Sci. USA.* 76:4230–4234.

Weiss, L. B., and R. E. Koeppe II. 1985. Semisynthesis of linear gramicidins using diphenyl phosphorazidate (DPPA). *Int. J. Pept. Protein Res.* In press.