EQUILIBRIUM BINDING CONSTANTS FOR THE GROUP I METAL CATIONS WITH GRAMICIDIN-A DETERMINED BY COMPETITION STUDIES AND Tl^+-205 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

JAMES F. HINTON, WILLIAM L. WHALEY, DIKOMA SHUNGU, ROGER E. KOEPPE II, AND FRANCIS S. MILLETT

Department of Chemistry, University of Arkansas, Fayetteville, Arkansas 72701

ABSTRACT The equilibrium binding constants of the Group I metal cations with gramicidin A in aqueous dispersions of lyso-PC have been determined using a combination of competitive binding with the Tl^+ ion and Tl-205 NMR spectroscopy. The values of the binding constants at 34°C are Li (32.2 M^{-1}), Na (36.9 M^{-1}), K (52.6 M^{-1}), Rb (55.9 M^{-1}), and Cs (54.0 M^{-1}). The equilibrium binding constant for the Tl^+ ion at this temperature is 582 M^{-1}. The relationships between the binding constants, the free energy of the binding process, and the cation selectivity of the gramicidin A channel are discussed.

INTRODUCTION

Voltage-gated ion-permeable channels in the membranes of excitable cells have the general characteristics of being cation selective for the monovalent cations and being able to maintain a high transport rate through the channel. The cation selectivity of the channel presumably depends upon interactions between binding groups on the channel and the permeant ion, while the high transport rate suggests that the ion-binding interactions must have rather small energies. The selectivity thus depends upon the relatively small differences in the heights of the peak energy barriers for the different ions (Hille, 1975). Gramicidin A is probably the best-characterized multibarrier, multioccupancy model channel system; however, there are still many questions about the binding selectivity and transport of this channel that are unanswered. Because of the apparent correlation between the ionic mobility of the Group I cations and the channel conductance at high concentrations of these ions, it was initially thought that the gramicidin A channel was simply a water-filled pore through which the ions moved together with their hydration spheres (Myers and Haydon, 1972; Hladky and Haydon, 1972). However, it is becoming clear now that the cation selectivity of gramicidin A is not just a matter of differences in ionic mobility (Eisenman et al., 1978, 1983, 1984; Andersen, 1983; Dani and Levitt, 1981; Urry et al., 1972, 1980; Finkelstein and Andersen, 1981; Levitt et al., 1978).

The movement of an ion through a gramicidin A channel involves five distinct steps: (*a*) diffusion through the aqueous phase up to the channel; (*b*) association with the channel entrance; (*c*) translocation through the channel interior; (*d*) dissociation from the channel; and (*e*) diffusion through the aqueous phase away from the channel (Andersen, 1983) (Fig. 1). The very informative presentation of the gramicidin dimer shown in Fig. 1 is that of Etchebest and Pullman (1985). Andersen (1983) has investigated the selectivity of the gramicidin A channel in terms of the relationship between the overall association rate constant for ion entry into the channel and the diffusion-controlled rate constant for movement through the aqueous phase up to the channel entrance for the Group I cations. Eisenman (1983, 1984) has calculated the energy profiles of the Group I cations with the gramicidin channel and finds significant differences in the binding affinities for the ions.

A number of calculations of apparent binding constants and determinations of the equilibrium binding constants for the Group I cations with the gramicidin channel have been reported. However, it is difficult to determine equilibrium constants for weakly bound systems, and there are large discrepancies in these values. Thallium-205, because of its relatively tight binding to gramicidin and its highly sensitive nuclear magnetic resonance (NMR) properties, has provided the opportunity for a determination of accurate equilbrium binding constants of Tl^+ to gramicidins A, B, and C (Hinton et al., 1986). Having the Tl^+-205 parameters (binding constant and bound chemical shift), we have applied the Tl-205 NMR technique to other ions by competitive displacement of bound Tl^+. The equilibrium binding constants for the Group I cations determined by this method appear to be the most consistent yet determined for gramicidin A.

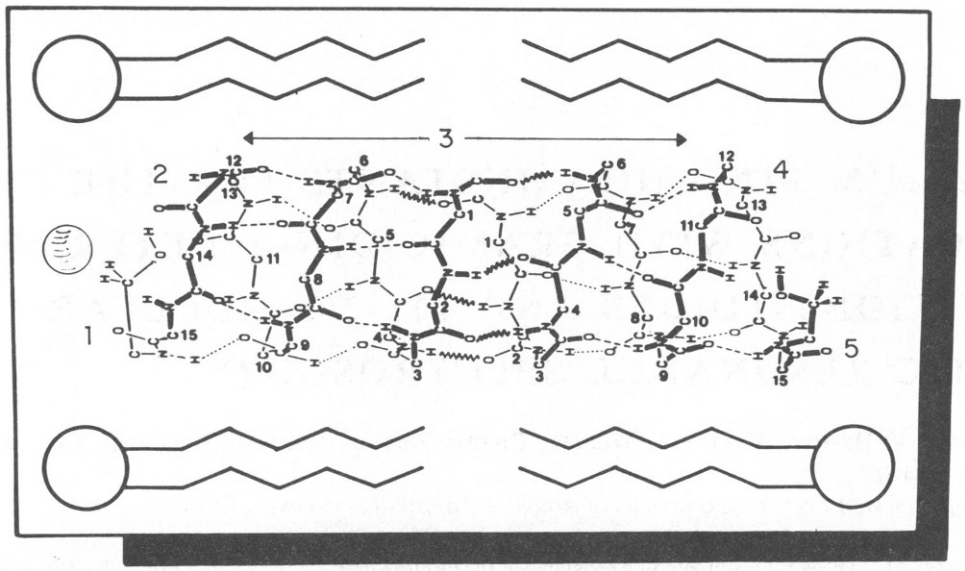

FIGURE 1 A schematic drawing of a gramicidin dimer within a lipid environment showing an approaching cation and the positions of the five steps involved in the transport process. The gramicidin dimer is that of Etchebest and Pullman (1985). (Permission to reproduce the gramicidin channel granted by Adenine Press).

MATERIALS AND METHODS

Gramicidin A was purified from commercial mixtures obtained from Sigma Chemical Company (St. Louis, MO) and U.S. Biochemical Corporation (Cleveland, OH) using previously described methods (Koeppe et al., 1985). The gramicidin A samples used for all of the experiments consisted of 92% gramicidin A and 8% gramicidin C. Gramicidin A was incorporated into lysophosphatidylcholine (lyso-PC) micelles using the method of Urry (Urry et al., 1979; Spisni et al., 1979; Hinton et al., 1982, 1985).

For the Li^+ and Na^+ solutions, aliquots of $TlNO_3$ and either $NaNO_3$ or $LiNO_3$ stock solutions were added to a solution of lyso-PC containing gramicidin A to yield the desired final concentration of each component (0.015 M $TlNO_3$, 0.1–0.4 M $LiNO_3$ or $NaNO_3$, and 0.005 M gramicidin A dimer). Initially, three to five 0.5-ml samples were prepared, each containing a different concentration of the alkali cation. After these samples were analyzed by Tl-205 NMR, additional samples were prepared by pairwise mixing of the initial samples.

For the Rb^+, Cs^+, and K^+ experiments, a stock solution of $TlNO_3$ (0.015 M), gramicidin A (0.005 M dimer), and lyso-PC (0.100 M) was first prepared. A portion of this stock solution was then added to solid Rb^+, Cs^+, or K^+ nitrate to yield a solution 0.4 M with respect to a particular nitrate salt (Tl^+ solubility limitations prevented the use of more concentrated stock solutions). Finally, a series of solutions ranging in concentration from 0.1 to 0.4 M, with respect to a particular K^+, Rb^+ or Cs^+ cation, was prepared by mixing the appropriate amounts of the 0.4 M solution and the original stock solution (free of Group I cation). Additional samples were prepared by pairwise mixing.

All determinations, except Rb^+, were performed using duplicate series of gramicidin samples. In addition, the effect of changing Tl^+ concentration was tested by using 10 mM $TlNO_3$ for one experiment with $NaNO_3$.

The Tl-205 NMR chemical shifts were recorded at 34°C as a function of varying Group I cation concentration (0.1 M–0.4 M) using a spectrometer (model HFX-90, Bruker Instruments, Inc., Billerica, MA) modified for pulsed-Fourier transform, (FT) operation with a temperature-controlled probe. Under the conditions of the experiments, the Tl^+ ions are in rapid exchange between free and gramicidin-bound states and only one resonance signal was observed for each solution. To correct for the lyso-PC–induced shift in the Tl-205 chemical shift, the shift produced by a lipid plus nitrate salt sample devoid of gramicidin was subtracted from the shift due to an equivalent gramicidin-containing sample (Hinton et al., 1986).

Under fast exchange conditions the single observed NMR signal is characterized by a chemical shift according to

$$X = p_i X_i, \tag{1}$$

where the X_i's are related to the chemical shifts of specific binding sites or interactions. The p_i's represent the fractional concentrations of the ions at given sites. In the case of a competition between ligands (i.e., different cations) for a common binding site on a macromolecule the fraction of the tighter binding ions at the binding site is given by the equation (Steinhardt, 1969; Forsen, 1982)

$$p_{Tl} = (n[Gr_2]\, K_{Tl}/(1 + K_{Tl}[Tl] + K_c[C]), \tag{2}$$

where $[Gr_2]$ is the effective gramicidin A dimer concentration, K_{Tl} is the equilibrium binding constant for the Tl^+ ion, [Tl] is the effective Tl^+ ion concentration (i.e., the thermodynamic activity), K_c is the equilibrium binding constant for a specific Group I cation, [C] is the effective concentration (activity) of the Group I cation, and n is the number of binding sites on the macromolecule, gramicidin A. Consequently, the observed chemical shift of the Tl^+-205 ion in the presence of a competing Group I cation (C) is given by

$$d_{obs} = (n[Gr_2]\, d_{Tl} K_{Tl})/(1 + K_{Tl}[Tl] + K_c[C]), \tag{3}$$

where K_c is, again, the equilibrium binding constant for the competing Group I cation and d_{Tl} is the bound chemical shift for the Tl^+–gramicidin A complex. The values of K_{Tl} (582 M^{-1}), d_{Tl} (127 ppm), n (1), and the effective gramcidin A concentration (2.47 mM) for an analytical concentration of 5 mM (or 1.5 mM for an analytical concentration of 3 mM) have been previously determined (Hinton et al., 1986). The nGr_2 product in the above equation was checked again by letting nGr_2 be an adjustable parameter in the curve-fitting process. The best-fit to the experimental data confirmed the previously determined value of n and the effective gramicidin A concentration. Since the amount of gramicidin C in the samples is very small and because the binding constant and bound chemical shift of the C analogue are very nearly the same as those of the A analogue, no correction has been made for the presence of the C

analogue. The effective concentration of the Tl^+ ion was calculated from the product of the activity coefficient of the Tl^+ ion and the analytical concentration. The activity coefficient of the Tl^+ ion was calculated using the Guggenheim equation (Guggenheim, 1935; Lewis and Randall, 1961). Experimental values of the activity coefficients were used to obtain the effective concentrations of the Group I cations (Harned and Owen, 1950). A nonlinear least squares fit of Eq. 3 to the experimental data (i.e., d_{obs} as a function of the varying concentration of the added Group I cation) gives the value of K_c for a specific Group I cation. The data were analyzed initially using a nonlinear least-squares analysis to get best-fit values for both K_c and Gr_2 . Since the same lyso-PC–gramicidin preparation was used for each ion, except Rb^+, and since the best-fit effective gramicidin concentration determined for each cation, and for Tl^+ alone (Hinton et al., 1986), were very nearly the same the best-fit effective gramicidin concentrations were averaged. The averaged value was then used for the final calculation of K_c for each cation.

RESULTS AND DISCUSSION

A typical data set and theoretical curve fit of the Tl^+-205 chemical shift as a function of the concentration of added Group I cation is shown in Fig. 2, where Li^+ is the competing cation. The sensitivity of the method is illustrated by the large change in chemical shift, even though the equilibrium binding constant of Li^+ is rather small. The equilibrium binding constants obtained by the competitive binding-Tl-205 NMR method are Li^+ (32.2 M^{-1}), Na^+ (36.9 M^{-1}), K^+ (52.6 M^{-1}), Rb (55.9 M^{-1}), and Cs^+ (54.0 M^{-1}). Because a nonlinear least-squares method was used in the analysis, it is important to consider the range in the binding constants at the 95% confidence level for each cation. The equilibrium binding constant range at the 95% confidence level is Li^+ (31–34 M^{-1}), Na^+ (36–38 M^{-1}), K^+ (51–56 M^{-1}), Rb^+ (55–58 M^{-1}), and Cs^+ (53–56 M^{-1}). Additional supporting evidence for this technique of determining equilibrium constants is the fact that in many enzyme systems it has been shown that the Tl^+ ion binds at least 10 times more strongly than the K^+ ion. The equilibrium binding constant results for gramicidin A are in agreement with this observation: $K(K^+) = 52.6\ M^{-1}$, $K(Tl^+) = 582\ M^{-1}$.

The results of the equilibrium binding constant measurements indicate that the binding constant of Li^+ is statistically different from that of Na^+. While the binding constant of K^+ is certainly different from that of Na^+, there appears to be no diffence, statistically, between the binding constants of K^+, Rb^+, and Cs^+. The free energy [$\Delta G = -RT(\ln K)$] of binding for each cation at 34°C is Li^+ (ΔG = -2.1 kcal/mol), Na^+ (ΔG = -2.2 kcal/mol), K^+

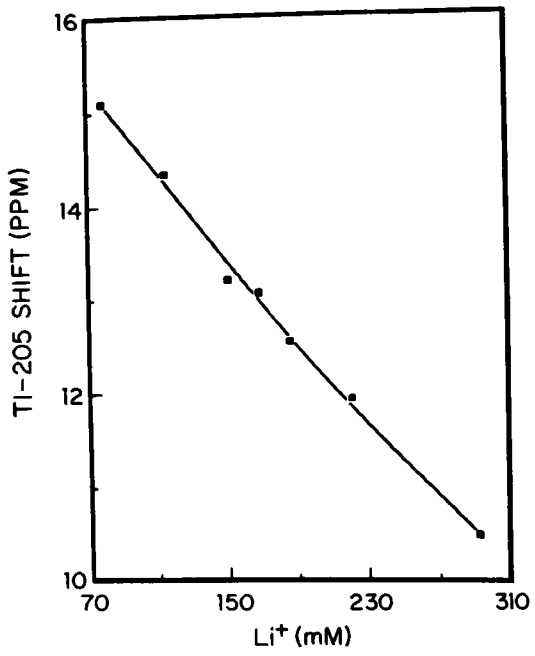

FIGURE 2 Tl-205 NMR chemical shift as a function of Li^+ concentration. Solid line represents theoretical fit to experimental data points.

TABLE I
EQUILIBRIUM BINDING CONSTANTS (M^{-1})
FOR THE TIGHT BINDING SITE OF GRAMICIDIN-A
IN BILAYERS AND MICELLES

Method	Ions				
	Li^+	Na^+	K^+	Rb^+	Cs^+
Tl-205 NMR (34°C)	32.2				
Li-7 NMR (30°C)*	14				
Conductance‡	9				
Conductance§	0.1				
Conductance‖	8.7				
Tl-205 NMR (34°C)		36.9			
Na-23 NMR (30°C)¶		100			
Na-23 NMR (30°C)**		70			
Na-23 NMR (36°C)‡‡		4			
Dialysis (23°C)§§		<30			
Conductance‡		20			
Conductance‖‖		1.43			
Conductance (25°C)¶¶		10			
Tl-205 NMR (34°C)			52.6		
Conductance‡			40.4		
Conductance‖‖			1.37		
Conductance‖			14.5		
K-39 NMR (30°C)***			8		
Tl-205 NMR (34°C)				55.9	
Dialysis (23°C)‖‖				<30	
Conductance‡				57.3	
Tl-205 NMR (34°C)					54
Conductance§					700
Conductance (25°C)‡					100
Cs-133 NMR (25°C)***					55

*Urry, 1983.
‡Eisenman, 1978.
§Eisenman, 1983.
‖Dani, 1981.
¶Urry, 1982.
**Venkatachalam, 1980.
‡‡Cornelis, 1979.
§§Veatch, 1980.
‖‖Levitt, 1978.
¶¶Russell, 1986.
***Urry, 1984.

(ΔG = -2.4 kcal/mol), Rb^+ (ΔG = -2.4 kcal/mol), and Cs^+ (ΔG = -2.4 kcal/mol). For Tl^+ the free energy of binding is ΔG = -3.8 kcal/mol.

A summary of the equilibrium binding constants for the Group I cations determined by several different techniques is contained in Table I. Binding constants for the Group I cations have previously been estimated by both equilibrium (i.e., NMR and dialysis) and kinetic (i.e., conductance) measurements. The variations in the values that have been reported (Table I) could be due to several factors, including the experimental approaches, the variations in the lipid environment of the gramicidin and in the temperature, the assumptions of particular transport models, and/or the methods of fitting theoretical curves to the experimental data. In the lyso-PC environment, circular dichroic spectroscopy shows gramicidin A to be in the conducting channel state (Hinton et al., 1986, Prasad et al., 1983). Equilibrium techniques require the often difficult choice (Hinton et al., 1986) between single-site and multiple-site models when selecting the best theoretical fit to the experimental data. Kinetic approaches to binding constants require additional assumptions regarding the elementary steps of the transport process. In fact, kinetic data can at best give an apparent binding constant that may or may not be equal to the equilibrium constant. In other words, kinetic and equilibrium experiments may not necessarily measure the same parameter.

Given the above considerations, it is notable that some of the values in Table I are in good agreement, though there are obvious discrepancies. For example, our equilibrium constants for K^+ (52.6 M^{-1}) and Rb^+ (55.9 M^{-1}) agree very well with one of the values derived from kinetic data for each of these ions (40 and 57 M^{-1}), although we disagree with an estimate from K-39 NMR. In the case of Li^+ and Cs^+, there are larger differences between the binding constants based on equilibrium as opposed to kinetic data (Table I). In fact, the trends for these two ions are opposite, as the conductance data give lower estimates for the Li^+ binding constant and higher estimates for the Cs^+ binding constant, when compared with NMR data. These systematic discrepancies, when substantiated by further measurements, could indicate a weakness in the theoretical models that are currently used to fit either the kinetic or the equilibrium data.

A large number of binding constants have been reported for Na^+ with gramicidin A. Although there are large variations, these values (Table I) tend to cluster about 30 M^{-1}. One of the values from a conductance study is unusually low (1.43 M^{-1}) and seems to be out of line with the others. Our estimate from the competitive displacement of the Tl^+ (37 M^{-1}) is intermediate among the various estimates ranging between 4 and 100 M^{-1} from Na^+=23 NMR. A particularly accurate kinetic estimate of 10 M^{-1}, based upon conductance data at five Na^+ concentrations and corrected for aqueous diffusion limitations, has recently been reported (Russell, E. W., L. B. Weiss, F. I. Navetta, R. E. Koeppe II, and O. S. Anderson, manuscript submitted for publication). While not very different considering the differences in the experimental approaches, our value of 37 M^{-1} and the conductance value of 10 M^{-1} for Na^+ are nevertheless considerably more different than the error estimate for either of these numbers. The small remaining discrepancy could be due to differences in lipid (Urban et al., 1980), temperature, or gramicidin concentration (> 6 orders of magnitude). However, the possible need to reconsider theoretical models of the transport process cannot be excluded.

The sequence in equilibrium binding constants that was obtained from the competitive binding–Tl-205 NMR method of $Li^+ < Na^+ < K^+ = Rb = Cs$ is the same as that obtained by Andersen (1983) with the estimates of the voltage-independent rate constant for the association of the cations with the channel. The rate constants at 25°C for gramicidin A in the lipid systems, diphytanoylphosphatidylcholine glycerylmonooleate, are shown in Table II (Andersen, 1983). Note that not only is the sequence the same for the equilibrium binding constants and the rate constants for association of the ion with the channel, but in both cases there is no significant difference in the binding constants or rate constants for the cations K^+, Rb^+, and Cs^+. Differences in temperature and lipid prevent one from calculating the rate constant for the dissociation process from the equilibrium binding constant and the rate constant for the association process.

The cation selectivity of the gramicidin channel is obviously a complex sum of effects. There is the selection due to ionic diffusion differences, selection in the binding process at the channel entrance, and selection within the channel due to differences in the interaction between the ions and the binding groups on the channel wall. The difference in overall conductance rate of the Group I cations through the gramicidin A channel depends upon these selectivity mechanisms. Consequently, one must be cautious in using just one of the selectivity mechanisms to describe the conductance sequence of the Group I cations. The total range of binding constants that we observe for the Group I cations is quite small, from 32 for Li^+ to about 55 M^{-1} for K^+, Rb^+, and Cs^+. Note that we compare all of the Group I cations under the same experimental conditions. The range is smaller than the corresponding range of

TABLE II
EQUILIBRIUM BINDING CONSTANTS (K_b) AND RATE CONSTANTS FOR ASSOCIATION (k_A) WITH THE CHANNEL

	Li	Na	K	Rb	Cs
K_b M^{-1}	32.2	36.9	52.6	55.9	54.0
*k_A (1/m-s) × 10^{-8}	>0.4	1	1.9	2.1	2.1
‡k_A (1/m-s) × 10^{-8}	>0.5	1.1	1.7	1.7	1.6

*Glycerlymonooleate lipid (Andersen, 1983).
‡Diphytanoylphosphatidylcholine (Andersen, 1983).

conductances for this series of ions. The results indicate that, although the ordering of the binding strengths is qualitatively the same as the ordering of the transport rates for the Group I ions, factors other than binding probably contribute to the selectivity of the channel.

The differences in the depth of the thermodynamic binding well at the channel entrance, even though small, do, however, play an important role in the overall transport mechanism of the channel. It may be that the well depth for K^+, Rb^+, and Cs^+ is just enough to assure stable ion capture at the channel entrance but is not too deep to prevent rapid transport. In the case of Li^+ and Na^+ the well may not be deep enough to assure adequate capture for optimal transport. In fact, according to Eisenman (1983), a high binding affinity enhances the permeability of the ion through the channel due to the relative energy position of the well with respect to the barrier height. Also note that thallium is in some sense a special case that should be treated separately from the Group I cations. Although Tl^+ binds much more tightly to gramicidin A and blocks the Group I cations in bionic experiments (Eisenman et al., 1977; Urban et al., 1980), the conductance of Tl^+ is approximately that of K^+ (i.e., not significantly larger due to high affinity for the channel or smaller due to a difficulty in exiting the channel). Instead, any difference in the off rate of Tl^+ seems to be balanced by corresponding differences in the on rate or the translocation rate. Thus, the correlation among the Group I cations, for which tighter binding leads to a higher conductance, cannot be extrapolated to the order of magnitude tighter binding of Tl^+. It is as though there is a rather broad optimum range of channel-ion affinities that balances the desired properties of selectivity and high transport rate.

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