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Dielectric Relaxation Studies of Ionic Processes in Lysolecithin-Packaged Gramicidin Channels

R. Henze, E. Neher*, T.L. Trapane**, and D.W. Urry**
Drittes Physikalisches Institut, Universitat Göttingen, D-3400 Göttingen 1, Germany

Summary. Dielectric permittivities have been determined for suspensions of lysolecithin packaged malonyl gramicidin channels over the frequency range of 5 kHz to 900 MHz and under conditions of approximately equimolar concentrations (~10 mM) of channels and salts. The salts were lithium chloride, sodium chloride and thallium acetate. A relaxation process unique to the thallium acetate-channel system was observed which on analysis gave rise to a relaxation time at 25° of 120 nsec. The permittivity data, as well as a comparison of binding constants, indicate that the relaxation process results from Tl^+ being bound within the channel and more specifically from an intrachannel ion translocation with a rate constant of approximately $4 \times 10^6 \text{ sec}^{-1}$ and with an energy of activation of less than 6.7 kcal/mole. These data compare favorably with data from conductance studies on planar bilayers and with ion and carbon-13 nuclear magnetic studies on the lysolecithin packaged malonyl gramicidin channels which combine to indicate that the relaxation process is due to the jump of the thallium ion across a central barrier.

Key words dielectric relaxation · gramicidin A · transmembrane channels · dielectric permittivity · intrachannel ion movements · relaxation times

Introduction

Interest in the gramicidin channel derives from many sources: (i) it exhibits high conductances, e.g., a single channel current of approximately 10^7 sodium ions/sec at 25 °C, 1 M NaCl and a 100 mV transmembrane potential [2, 3]; (ii) it exhibits very significant ion selectivity, being essentially impermeable to anions and divalent cations and exhibiting substantial selectivity among monovalent cations with permeability ratios of $H^+(150) > NH_4^+(8.9) > Cs^+(5.8) > Rb^+(5.5) > K^+(3.9) > Na^+(1.0) > Li^+(0.33)$ as early reported by Myers and Haydon [18]; and (iii) it exhibits additional properties similar to those of physiological channels, e.g., the magnitude of the single-channel conductance (about 10 pS), saturation and maxima in conductance as a function of ion concentration, concentration dependence of permeability ratios, and ion competition and block [5, 19].

The structure of the gramicidin channel is now well accepted to be the head-to-head dimerization of two monomers [1, 24, 27, 34] which are each in a single stranded β-helical conformation [20, 21, 24–28]. The resulting structure has a two-fold symmetry axis perpendicular to the channel (helix) axis, a 4-Å diameter channel and a length of about 26 Å [16, 26, 28]. This relatively simple structural picture engenders further interest due to additional subtleties that relate to the observation that the most probable single-channel conductance state is accompanied by numerous less probable lower conductance states [4, 14] which appear to be lipid dependent [4]. It has been proposed that these result from numerous combinations of different side chain rotamer states arising from the interactions of the bulky side chains and altering the energetics of the peptide librations required for ion coordination [31]. The large side chains are apparent in the primary sequence of gramicidin A [10, 22], i.e., HCO-L·Val_1-Gly_2-L·Ala_3-D $-Leu_4$-L·Ala_5-D·Val_6-L·Val_7-D·Val_8-L·Trp_9-D ·Leu_{10}-L·Trp_{11}-D·Leu_{12}-L·Trp_{13}-D·Leu_{14}-L·Trp_{15} $-NHCH_2CH_2OH$, where they are seen to cluster at the carboxyl (ethanolamine or tail) end of the molecule. Interestingly, this is the end where the rate limiting barrier for transport is thought to occur [7, 32, 33].

It has recently been demonstrated that gramicidin channels can be incorporated into phospholipid structures made of L-α-lysolecithin [29, 30]. Using sodium-23 nuclear magnetic resonance (NMR), the phospholipid packaged gramicidin channel was found to bind sodium with two binding constants, for the tight site, $K_b^t \simeq 70 \text{ M}^{-1}$, and for the weak site, $K_b^w \simeq 1 \text{ M}^{-1}$ [32, 33]. Off rate constants were also

* *Permanent address:* Max-Planck-Institut für Biophysikalische Chemie, Postfach 968, D-3400 Göttingen, Germany

** *Permanent address:* Laboratory of Molecular Biophysics, University of Alabama in Birmingham, Birmingham, Alabama 35294.

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reported for both the tight and weak sites. The resulting four rate constants and a parametrized fifth rate constant, representing an intrachannel ion movement, were found by means of Eyring rate theory to satisfactorily calculate the single-channel currents for 50, 100, 150 and 200 mV transmembrane potentials and over several decades of NaCl concentration [32, 33]. The fitted rate constant for the central barrier was $3.2 \times 10^6 \text{sec}^{-1}$. As this intrachannel ion translocation was not directly determinable from cation NMR and as this is the only adjustable rate constant, it becomes of particular interest to obtain independent measures of the rate constant for intrachannel ion movements.

In the present effort dielectric relaxation studies are reported on N,N′-(dideformyl gramicidin A)-malonamide (also referred to as malonyl bis desformyl gramicidin or simply malonyl gramicidin) incorporated into lysolecithin phospholipid structures for the Li^+, Na^+ and Tl^+ ions wherein a relaxation process is observed for Tl^+ which is reasonably taken to result from an intrachannel ion movement.

Materials and Methods

Lysolecithin Packaging of Malonyl Gramicidin Channels

For both reference and for subsequent channel incorporation, micelles were formed by dissolving L-α-lysolecithin (Avanti Biochemicals, Birmingham, Alabama, lot No. LPC-18) in 10 mM salt solutions each of $TlO_2C_2H_3$, NaCl and LiCl in D_2O and then sonicating three times for 3 min each time in a Heat Systems cell disruptor equipped with a cup horn accessory. For channel incorporation, malonyl-bis-desformyl Gramicidin A was then added as a lyophilized powder to the micellar suspension to give an 11 mM concentration of malonyl gramicidin and a 1:30 molar ratio with the phospholipid. The samples were then vortexed and sonicated two times for six minutes each time to disperse the peptide. Heat incorporation was carried out in a thermostatted bath at 70 °C for 36 hr with sonication at intervals to break up the large particles which formed during incubation. The samples were then centrifuged at $3000 \times g_{max}$ for 30 min at room temperature to remove the denser particles. The supernatants, which remained quite cloudy at this relatively higher concentration of malonyl gramicidin, were directly used in the subsequent characterizations. The state of incorporation for each sample was checked by taking an aliquot of the supernatant, diluting with distilled water, and determining the circular dichroism spectrum from 300 to 185 nm. The spectrum characteristic of complete channel formation [17, 29] was observed for all samples. Figure 1 shows the circular dichroism spectrum for the sample cntaining 10mM $TlO_2C_2H_3$. In order to determine the malonyl gramicidin concentration, duplicate aliquots of each sample were lyophilized, redissolved in methanol, and the absorption spectra taken. Using a molar extinction coefficient of 2.25×10^4 at 282 nm the malonyl gramicidin (channel) concentrations were found to be 8.7 mM for the $TlO_2C_2H_3$, 9.4 mM for the NaCl and 9.8 mM for the LiCl samples. The samples were also examined in the electron microscope using the technique of negative staining where under these conditions of higher channel concentrations, large multilamellar structures were observed rather than micelles of slightly increased radii.

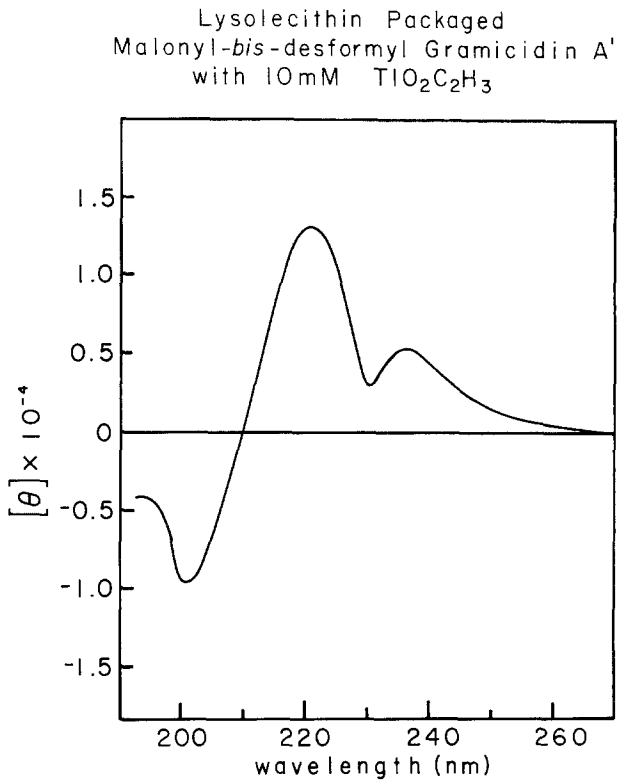

Fig. 1. Circular dichroism spectrum of the lysolecithin/malonyl gramicidin/thallium acetate system demonstrating complete incorporation and conversion to the channel state

Dielectric Measurements

The real part $\varepsilon'(\nu)$ and the imaginary part $-\varepsilon''(\nu)$ of the complex dielectric permittivity,

$$\varepsilon(\nu) = \varepsilon'(\nu) - i\,\varepsilon''(\nu) \tag{1}$$

were measured over the frequency range, $\nu = 5$ kHz to 900 MHz, using three different methods. Within the range $\nu = 1$ to 100 MHz, the measuring cell was a coaxial line/circular waveguide transition, used far below the cutoff-frequency of the waveguide. The waveguide was filled with the dielectric sample. This configuration can be described as a coaxial line terminated by a capacitor, which is filled with the dielectric. The capacitance and conductance of the cell were measured with a Boonton 33 D/1 RF-bridge. This type of cell was used with earlier measurements and is described in other publications [11–13]. For the frequencies $\nu = 10$ to 900 MHz, a transmission method was used. The measuring cell was a slab line with an inner conductor gap, which was filled with the dielectric sample. The magnitude and phase of the transmission coefficient of the cell were measured with a Rohde & Schwarz Vektorvoltmeter ZPU. This method and the cell are described elsewhere [9]. In the lower frequency range, $\nu = 5$ to 500 kHz, the series capacitance and conductance of the slab line cell were measured with a Boonton B75C bridge. Measurements were carried out at different temperatures covering the range of 25 to 55 °C.

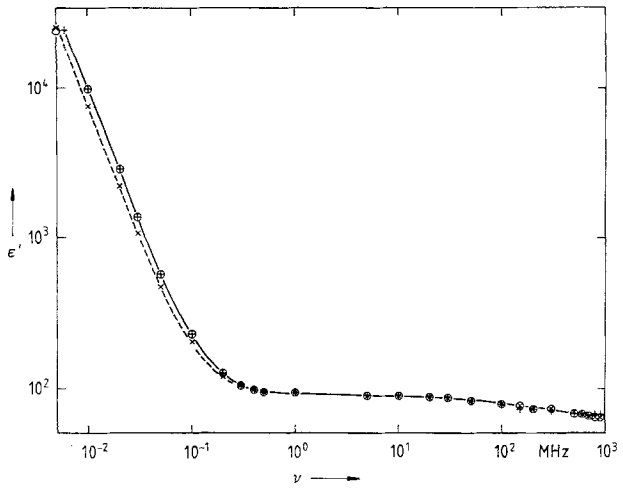

Fig. 2. Log/log-plot of the real part ε' of the dielectric permittivity against frequency ν for the lysolecithin solutions at 25 °C. +—— lysolecithin/$TlO_2C_2H_5$ (LTl), ×------ lysolecithin/LiCl (LLi), ○— — lysolecithin/NaCl (LNa)

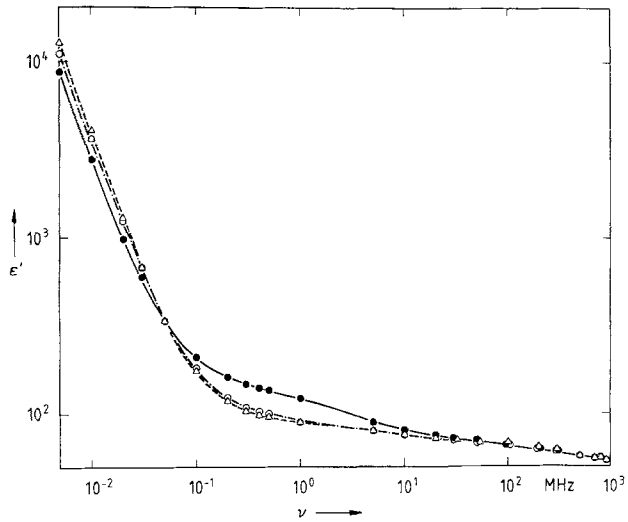

Fig. 3. Log/log-plot of the real part ε' of the dielectric permittivity against frequency ν for the gramicidin/lysolecithin solutions at 25 °C. ●—— gramicidin/lysolecithin/$TlO_2C_2H_5$ (GLTl), ○——·—— ·gramicidin/lysolecithin/LiCl (GLLi), △------ gramicidin/lysolecithin/NaCl (GLNa)

Results

Frequency Dependence of ε' and ε''

In Figs. 2 and 3, ε' is plotted against the frequency, ν, for the lysolecithin and the gramicidin/lysolecithin solutions, respectively. At low frequencies ($5\,kHz \leqq \nu \lesssim 100\,kHz$), ε' decreases steeply with increasing ν due to polarization effects at the cell electrodes. The weak decrease of ε' at frequencies above 10 MHz indicates a relaxation process at frequencies between approximately 100 and 1000 MHz. At 1000 MHz, ε' approaches the permittivity of the diluted solvent. The data of the three lysolecithin solutions are nearly identical (*see* Fig. 2) except for frequencies $\nu \lesssim 500\,kHz$ where the difference in ε' reflects different conductivities of the solutions. Also the two dispersion regions at low and high frequencies are clearly separated by a region of nearly constant ε' between $\nu \simeq 1$ and 20 MHz.

The gramicidin/lysolecithin solutions with added LiCl (GLLi) and NaCl (GLNa) (*see* Fig. 3) show a monotonic decrease of ε' for the range $\nu \simeq 1$ to 1000 MHz. With the gramicidin/lysolecithin/$TlO_2C_2H_3$ solution (GLTl) an additional dispersion step is superimposed in the $100\,kHz \lesssim \nu \lesssim 10\,MHz$ range. This is shown more clearly in Fig. 4, where ε' is plotted against ν with a higher resolution in the ε' scale for the GLTl solution in comparison with the

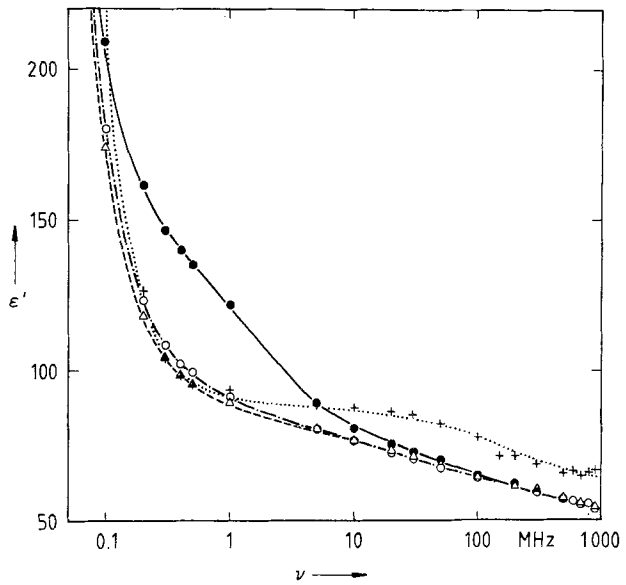

Fig. 4. Real part ε' of the dielectric permittivity plotted against $\log(\nu)$ for frequencies $\nu = 0.1$–1,000 MHz at 25 °C. The curves are fitted according to Eqs. (2) and (3) to the data points. ●—— gramicidin/lysolecithin/$TlO_2C_2H_5$ (GLTl), ○————gramicidin/lysolecithin/LiCl (GLLi), △------ gramicidin/lysolecithin/NaCl (GLNa), +... lysolecithin/$TlO_2C_2H_5$ (LTl)

GLLi, GLNa and LTl solutions. (It should be noted that the LTl curve above 0.5 MHz is representative for LLi and LNa, too [*see* Fig. 2])!

In Fig. 5, ε'' is plotted against ν for all solutions. ε'' decreases with increasing ν by 5 orders of magnitude. The ε'' (ν) relation is nearly linear in the log/log-plot and reflects mainly the conductivity contri-

bution to ε''. The differences between the curves of the different solutions indicate different conductivities.

Calculation of Relaxation Parameters

A relaxation spectrum similar to that found in the LTl, LLi, LNa solutions in the upper frequency range ($\nu \approx 10$–900 MHz) was previously observed with earlier measurements on an aqueous lysolecithin solution [15]. It could be described by a Cole-Cole relaxation function. To the present data was fitted a spectral function consisting of a sum of Cole-Cole functions, of a term describing the conductivity contribution to ε'' and of a term describing the electrode polarization. Two Cole-Cole functions and the conductivity term

$$\varepsilon_D(\nu) = \varepsilon_\infty + \frac{\Delta\varepsilon_1}{1+(i2\pi\nu\tau_1)^{1-\alpha_1}} + \frac{\Delta\varepsilon_2}{1+(i2\pi\nu\tau_2)^{1-\alpha_2}} + \frac{\sigma}{i2\pi\nu\varepsilon_o} \quad (2)$$

were fitted to the data of the GLTl solution, while with the other solutions a single Cole-Cole function was used ($\Delta\varepsilon_2 = 0$). ε_∞ is the high frequency limit of the relaxation function and represents the solvent contribution to the permittivity. $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$ are the relaxation amplitudes of ε'_D, and τ_1 and τ_2 are the mean relaxation times. α_1 and α_2 describe the relaxation time distributions. σ is the conductivity, and ε_0 is the vacuum permittivity. The contribution of the electrode polarization was considered as a frequency-dependent capacitance, $C = C_0 \nu^{-m}$ [23], in series with the capacitance, C_M, of the empty cell. The relation between the measured permittivity, ε, and the permittivity $\varepsilon_D(\nu)$ of the dielectric is given by

$$\varepsilon(\nu) = \frac{\varepsilon_D(\nu)}{1+\varepsilon_D(\nu)\cdot R\cdot(\nu/10^6\,\mathrm{s}^{-1})^m}, \quad \text{with } R = \frac{C_M}{C_0}. \quad (3)$$

This function, with $\varepsilon_D(\nu)$ inserted according to Eq. (2), was fitted to the measured permittivity data by varying the parameters ε_∞, $\Delta\varepsilon_1$, τ_1, α_1, $\Delta\varepsilon_2$, τ_2, α_2, σ, R and m. Examples for the agreement of the measured data and the fitted functions are given in

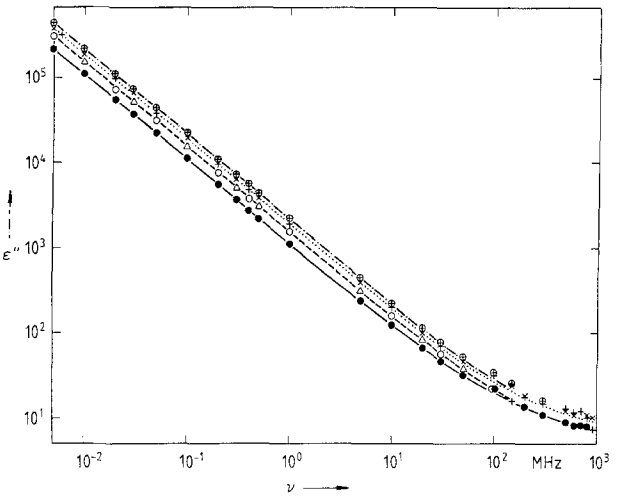

Fig. 5. Log/log-plot of the negative imaginary part ε'' of the dielectric permittivity against frequency ν for the gramicidin/lysolecithin and the lysolecithin solutions at 25 °C. ●—— gramicidin/lysolecithin/$TlO_2C_2H_5$ (GLTl), ○------ gramicidin/lysolecithin/LiCl (GLLi), △---- gramicidin/lysolecithin/NaCl (GLNa), +... lysolecithin/$TlO_2C_2H_5$ (LTl), ×..... lysolecithin/LiCl (LLi), ⊕————lysolecithin/NaCl (LNa). All solutions were measured at the same frequencies. For a better survey, some data points are not plotted

Table 1.

Solution (short notation)	T [°C] ±0.2 °C	σ [Ω^{-1} m^{-1}] ±4%	m ±6%	R [10^{-6}] ±15%	$\Delta\varepsilon_2$ ±30%	τ_2[nsec] ±50%	α_2 ±0.05	$\Delta\varepsilon_1$ ±6%	τ_1[nsec] ±20%	α_1 ±6%	ε_∞ ±8%
GLTl	25.0	0.061	0.33	1.0	30	122	0	108	64	0.71	39
GLTl	36.4	0.077	0.35	0.98	35	47	0	79	15	0.71	36
GLTl	46.4	0.094	0.36	1.0	36	41	0	55	6	0.62	41
GLTl	55.6	0.109	0.39	1.0	36	43	0	44	4.5	0.51	44
GLLi	25.0	0.085	0.34	0.73	–	–	–	97	52	0.77	34
GLLi	35.5	0.106	0.33	0.77	–	–	–	61	9	0.67	41
GLLi	47.2	0.13	0.32	0.69	–	–	–	38	4.6	0.51	48
GLNa	25.0	0.086	0.27	0.58	–	–	–	120	86	0.83	24
GLNa	34.7	0.10	0.25	0.51	–	–	–	85	27	0.77	34
GLNa	47.5	0.13	0.26	0.46	–	–	–	47	4.5	0.63	42
LTl	25.0	0.108	0.14	0.49	–	–	–	33	0.68	0.37	56
LLi	25.0	0.107	0.16	0.41	–	–	–	40	0.73	0.43	52
LNa	25.0	0.123	0.23	0.51	–	–	–	37	0.58	0.41	53

G stands for gramicidin and L for lysolecithin, and the others are the standard symbols for the elements.

Fig. 4. The results for the fitted parameters are given in Table 1.

Discussion

Several different polarization processes can contribute to the dielectric relaxation spectrum observed in the upper frequency range ($\Delta\varepsilon_1$, τ_1, α_1, $\nu \gtrsim 10$ MHz): i) the diffusive reorientation of the zwitterionic phosphatidylcholine groups of the lysolecithin molecules, ii) the diffusive motion of counterions on the surface of the phospholipid structure, and iii) the lipid structure limited drift motion of the ions in the solvent (i.e., the Maxwell-Wagner effect).

It should be noted that for the GLTl, GLLi and GLNa solutions the fit of a Cole-Cole function ($\Delta\varepsilon_1$, τ_1, α_1) is not necessarily indicated by the frequency dependence of the data between $\nu=1$ to 1,000 MHz. This function yields a good fit of the data within the limits of experimental error. However, the physical meaning of the parameters $\Delta\varepsilon_1$, τ_1, α_1 and ε_∞ is doubtful, because the fitted function extends to frequencies far beyond the range $\nu=1$ to 1,000 MHz ($\alpha_1 \simeq 0.5$–0.8).

The additional relaxation term ($\Delta\varepsilon_2$, τ_2, α_2) in the 0.1–10 MHz range only occurs in the gramicidin/lysolecithin solution containing thallium acetate. This solution differs from the others in having a considerable amount of Tl^+-ions trapped within the gramicidin channels. This is due to the much larger binding constant for Tl^+ ($\sim 1\times10^3\ M^{-1}$) than for Li^+ ($\sim 20\ M^{-1}$) and Na^+ ($\sim 70\ M^{-1}$) as determined by NMR studies [32 and *unpublished data*]. These tight binding constants agree within a factor of 2 or 3 with those obtained by Eisenman et al. [5, 6] from conductance studies. That the Tl^+ ions are trapped within the channels is also indicated by the relatively small conductivity of the GLTl solution in comparison with the others. Therefore the relaxation spectral term in the 0.1–10 MHz band is the result either of thallium ion movements within the channel or of channel reorientation. As it is quite unlikely that the gramicidin containing multilamellar structures would move with such a rapid rotational correlation time and even less so that the channel could rotate on an axis perpendicular to the channel axis within the micelle at such rates, the relaxation time at 25 °C with a τ_2 of 120 nsec is reasonably taken to be the average time between two jumps.

A theoretical description of the dielectric polarization by an ion jump process between two equivalent sites has been given by several authors [8, 35]. The polarization contribution of a channel is $(ed/2)^2 \cos^2\theta E/kT$. e is the elementary charge, d the distance between the free energy minima in the channel (which is the length of the ion jump), θ the angle between the direction of the channel axis and the applied field, E the field strength, k Boltzmann's constant, and T temperature. If one assumes that the channels are fixed (i.e., the reorientation time of the channel is much longer than the average time between two jumps of the Tl^+-ion within the channel) and randomly oriented, the permittivity contribution of the channels is

$$\Delta\varepsilon = \frac{CL(ed/2)^2}{\varepsilon_0 2kT}. \tag{4}$$

C is the molar concentration of the channels occupied by a Tl^+-ion and L is Avogadro's number. With $C=8.7\times10^{-3}$ mol/liter, $d=23$ Å[1] and $T=298.2$ K, Eq. (3) yields $\Delta\varepsilon\simeq 3$. This value is distinctly smaller than the measured value $\Delta\varepsilon_2=30$. The difference can be caused by parallel orientation correlation of the channels, possibly due to aggregation. This interpretation requires about 10 neighboring channels parallel oriented with correlated jumps of the ions.

The magnitude, E_a, of the potential energy barrier between the two binding sites of the channel can be estimated from the temperature dependence of the relaxation time. According to reference [8] the relaxation time is

$$\tau = \frac{\pi}{\omega_0} e^{\frac{E_a}{kT}}. \tag{5}$$

ω_0 is the angular oscillation frequency of the ion in the potential minimum at one binding site. The variation of τ_2 with T in Table 1 yields $E_a < 6.7$ kcal/mol. A more precise value of E_a is made difficult because of the poor fit of the temperature dependence of τ_2 (*cf.* Table 1) to the exponential law of Eq. (5). This is due to experimental uncertainty, particularly due to the limited number of data points between 1 and 10 MHz.

The finding of a relaxation process defining a rate over a central barrier is consistent with other studies on the lysolecithin packaged gramicidin channels. Carbon-13 nuclear magnetic resonance studies using selective carbon-13 enrichment of carbonyl carbons have shown that malonyl carbonyl carbons are not perturbed even by a 30-fold excess of Tl^+ concentration over the channel's concentration [31], nor are the formyl and Val_1 carbonyl

[1] The position of the Tl^+ binding site in lysolecithin packaged gramicidin channels has been determined, using a series of synthetic $1-{}^{13}C$ amino acid gramicidin A molecules, to be at the Trp_{11} carbonyl oxygen which gives a length, d, of about 23 Å (D.W. Urry, K.U. Prasad & T.L. Trapane, *Proc. Natl. Acad. Sci. USA (in press)*

carbons perturbed (D.W. Urry et al., *in preparation*). The carbonyl carbons of the tryptophans at positions 9, 11 and 13, however, are perturbed at equimolar concentrations of Tl^+ and channels with the largest perturbation being experienced by the Trp_{11} carbonyl (D.W. Urry et al., *in preparation*). This indicates that there is localized binding and a central barrier.

The mean relaxation time, τ_2, and the associated energy barrier are also consistent with other data on gramicidin channel transport. Because the nuclear spin of Tl^+ is 1/2 (rather than 3/2 as in the case for sodium), NMR derived rate constants, which could be used to calculate the single-channel currents and thereby to obtain a fitted value for the central barrier rate constant, have not been obtained. Thus the direct comparison of the Tl^+ rate constants is not possible, but a useful comparison with the values obtained for sodium is possible. Using the NMR-derived rate constants for Na to calculate the single-channel currents, the value of k_{cb} (Na) of $3.2 \times 10^6\,\mathrm{sec}^{-1}$ gave the optimal fit [32]. This defines a reasonable magnitude for k_{cb}. Since $\tau_2 \simeq 120$ nsec, $k_{cb}(\mathrm{Tl}) = 1/2\tau_2 \simeq 4 \times 10^6\,\mathrm{sec}^{-1}$ which is a consistent value for the rate constant. Additionally, the rate limiting energy barrier for sodium transport through the channel is 7.3 kcal/mole for dioleoyl lecithin membranes [3], which is the same as the exit barrier for the lysolecithin packaged channels [32]. This means that the central barrier for the sodium ion should be less than 7.3 kcal/mole such that $E_a < 6.7$ kcal/mole for Tl^+ is again a consistent value.

Thus it is reasonable to conclude that dielectric relaxation studies can be used to characterize intrachannel ion translocation when the binding constants are sufficiently large, e.g., $\sim 10^3\,\mathrm{M}^{-1}$, and specifically that the relaxation process with relaxation time of 120 nsec is relevant to the jump of Tl^+ across the central barrier.

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