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Potassium-39 NMR of K^+ Interaction with the Gramicidin Channel and NMR-Derived Conductance Ratios for Na^+, K^+ and Rb^+

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Summary. A potassium-39 NMR study of potassium ion interaction with the gramicidin transmembrane channel in phospholipid bilayers at high ion activity is reported which allows determination of a weak binding constant, $K_b^w \simeq 8.3$/M, and an off-rate constant for the weak site, $k_{off}^w \simeq 2.6 \times 10^7$/sec. These values are interpreted with the aid of additional NMR data as the binding constant for formation of the doubly occupied channel state and the rate constant for an ion leaving the doubly occupied state. Considering the singly occupied channel state for the potassium ion to be "electrically silent" at 1 molar ion activity, as with the sodium ion, the single-channel conductance for 100 mV and 30°C calculated to be 29 pS, and using the same approximation with previous NMR results on the sodium and rubidium ions, reasonable conductance ratios were calculated. Further experimental estimates of the other three constants with the experimental location of binding sites and Eyring rate theory to introduce voltage dependence allowed a more complete calculation of the two-site channel. The single-channel conductance for potassium ion is calculated to be 24 pS at 1 M activity and 26 pS at 0.6 M activity, which compares for diphytanoyl phosphatidylcholine membranes to an experimental most probable single-channel conductance of 25 pS and a mean channel conductance of 20 pS. The calculated conductance ratios using NMR-derived constants were $\gamma(K)/\gamma(Na) = 2.0$ and $\gamma(Rb)/\gamma(Na) = 4.3$. These results are close to the experimental values and provide further basis for the use of NMR of quadrupolar ions to provide information on the ionic mechanism of channel transport.

Key Words quadrupolar nuclear magnetic resonance · rotational correlation times and off-rate constants · potassium-39 NMR · gramicidin channel transport · ion transport specificity · electrophysiology without electrodes

Introduction

Several years ago [20, 21] sodium-23 NMR was first used to estimate binding and rate constants for sodium ion interaction with gramicidin channels in phospholipid bilayers formed on incubation of gramicidin with L-α-lysolecithin (also referred to as lysophosphatidylcholine or lyso-PC). Specifically two binding constants, K_b^t and K_b^w, were obtained for tight, t, and weak, w, binding sites, and the off-rate constants, k_{off}^t and k_{off}^w, were also obtained. This provided four of the five rate constants required to calculate the single-channel currents for a two-site, twofold symmetric, single filing channel with Eyring rate theory being used to calculate the voltage dependence and with known locations for the binding sites [14]. The fifth rate constant, k_{cb}, is for the rate of ion translocation between the two symmetrically located binding sites. With a reasonable value for this central barrier rate constant, the sodium ion single-channel currents could be calculated over significant ranges of ion activity and applied potential. For the malonyl dimer of gramicidin, the fitted value of k_{cb} for sodium ion was found to be essentially the same as that experimentally determined by dielectric relaxation measurements for the thallium ion [6]. Subsequently, two binding constants for the lithium ion interaction with the gramicidin channel have been determined using lithium-7 NMR [16], and more recently two binding constants have been estimated for cesium ion using cesium-133 NMR [15], and K_b^w and k_{off}^w have been estimated for rubidium ion using rubidium-87 NMR [19].

In the present effort K_b^w and k_{off}^w are estimated by means of potassium-39 NMR, the former using longitudinal relaxation time data and the latter using an appropriate ratio of longitudinal to transverse relaxation times. This binding and rate constant are sufficient to provide a reasonable estimate of the potassium ion single-channel current when considering only the singly (XO and OX) and doubly occupied (XX) states of the channel and the entrance-exit barrier on the positive side of the membrane. When equivalent data from the rubidium-87 and sodium-23 studies are used, it becomes possible similarly to calculate these single-channel currents and to obtain conductance ratios of potassium ion and of rubidium ion with respect to sodium ion. These ratios compare favorably with literature values. More complete single-channel current calculations are also reported using the full formalism for this

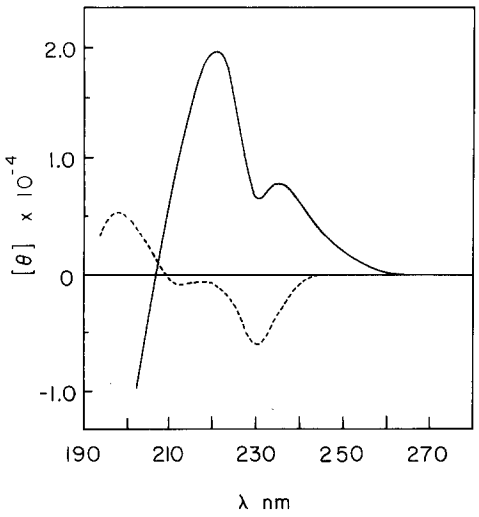

Fig. 1. Circular dichroism spectra of gramicidin in association with L-α-lysolecithin in water. The dashed curve is the initial associated nonchannel state. The solid curve demonstrates the conversion after heat incubation to the channel state. This is one of the criteria used to verify that the proper channel state has been achieved prior to initiation of NMR studies

two-site channel [20] and reasonable NMR-derived approximations to the other constants to which the currents are obviously not so sensitive. Again the calculated currents compare favorably with the experimental currents and conductance ratios. This effort provides substantial support for the quadrupolar ion NMR analyses, which give rise to the binding and rate constants and to the use of NMR-derived values to develop ionic mechanisms of channel transport.

Materials and Methods

The sample used for the potassium-39 NMR studies was naturally occurring gramicidin (ICN Nutritional Biochemicals Corporation, Cleveland, OH), which is a known mixture of Phe[11]GA (9%), Tyr[11]GA (19%) and GA (72%) and which was lyophilized and used without further purification. The procedures for channel incorporation and for determination of channel concentration were as previously described [6, 15, 16]. Verification of the ion-interacting channel state for this preparation was achieved by observing the circular dichroism spectrum and by determining the ion NMR chemical shift at 30°C. The CD spectrum for the preparation used in the potassium-39 NMR studies is given in Fig. 1. The potassium-39 chemical shift for 0.3 mM channels and 100 mM KCl was 14.7 ppm upfield from 100 mM KCl in ^2H_2O. These results indicate essentially complete channel formation [6, 15, 16]. The potassium ion titrations were carried out by addition of dry KCl (J.T. Baker Chemical Co., Phillipsburg, N.J., analytically certified ULTREX Lot 307081) to a fixed volume of sample in the NMR tube.

The potassium-39 nuclear magnetic resonance studies were performed on a JEOL FX-100 spectrometer with a multinuclear probe operating at 4.65 MHz. As the potassium-39 signal was difficult to observe at low ion concentration in the presence of 3 mM channels, the data were collected at a channel concentration of 0.3 mM by dilution of the 3 mM sample. Longitudinal relaxation times were measured by the inversion recovery (180°-τ-90°) method using from six to nine partially relaxed spectra accumulated at different pulse intervals, τ, for each T_1 value obtained at each ion activity. The rate for the free potassium ion, R_{1f}, was determined for 100 mM KCl in ^2H_2O, and the contribution of the lipid to the potassium ion relaxation was determined by carrying out a titration on lysolecithin micelles in ^2H_2O alone at the same lipid concentration which was used in the channel preparation. All of the potassium-39 measurements were performed at 30 ± 2°C.

In order to obtain ion activities from ion concentrations, activity coefficients for KCl are needed. Though experimental values of ion activities for various ionic salts are available in the literature [5], for our purposes an analytical expression for the activity coefficients is desirable to enable automatic corrections for activities in the fitting procedures routinely employed in these studies. The experimental activities are known to be well-represented by a 4-parameter Guggenheim equation shown in Eq. (1) where m is the molality. The values of the four parameters were optimized using a quasi-Newton algorithm to reproduce experimental KCl activity coefficients over the concentration range of 0.5 mM to 3 M. The values of the parameters obtained for KCl are shown in Eq. (1).

$$\log(\text{activity coefficient}) = \frac{-0.51129m^{1/2}}{1 + 1.2998m^{1/2}} + 0.00098515m + 0.0030902m^2. \quad (1)$$

This equation was employed in the plotting of data in Fig. 3 below.

Results

A stack of ten potassium-39 NMR inversion recovery (180°-τ-90°) spectra with pulse intervals, τ, labeled is given in Fig. 2 for 0.3 mM channels, 1 M KCl (0.605 molar activity) and 30°C. The experimental T_1 is 15.3 msec. When such studies are carried out over a range of concentrations, the data may be plotted in terms of the inverse of the excess longitudinal relaxation rate [9]. From the longitudinal relaxation rate, $R_1 = 1/T_1$, obtained for the channel sample is subtracted the longitudinal relaxation rate in the presence of an equal amount of lyso-PC, i.e., $R_1(l) = 1/T_1(l)$ where in this case $T_1(l) = 55$ msec. In the plot of the inverse of the excess longitudinal relaxation rate as a function of molar ion activity in Fig. 3, the data is found to fit a straight line and the reciprocal of the negative X-axis intercept gives the binding constant for the ion interaction when the ion concentration is much greater than the site concentration. The intercept gives a binding constant, K_b^w, of 8.3/M (all binding constants

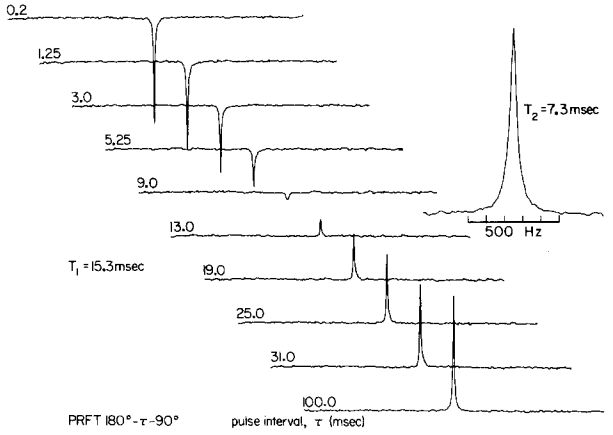

Fig. 2. Potassium-39 NMR longitudinal relaxation time (T_1) study of 1 M (0.6 molar activity) KCl in the presence of 0.3 mM gramicidin channels incorporated with L-α-lysolecithin into phospholipid bilayers. These are inversion recovery (180°-τ-90°) partially relaxed Fourier transformed spectra with the pulse intervals, τ, indicated at left. The inset is an enlargement of the totally relaxed spectrum which is used to obtain the value of the transverse relaxation time, $T_2 = 1/\pi\nu_{1/2}$

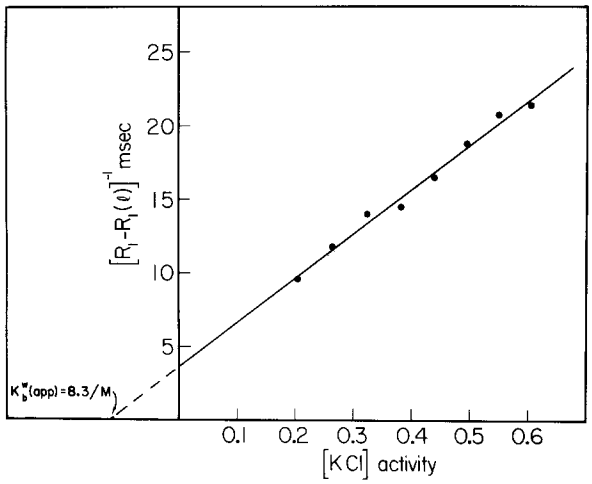

Fig. 3. Excess longitudinal relaxation rate plot for potassium-39 in the presence of 0.3 mM gramicidin channels incorporated with L-α-lysolecithin into phospholipid bilayers. The reciprocal of the negative X-axis intercept gives a binding constant of 8.3/M when the X-axis is a plot of ion activities

considered in this paper have been determined using ion activity).

The next quantity obtainable from the data is an off-rate constant at high ion activity. This rate constant is derived from the ion correlation time, τ_c. Following Rose and Bryant [12], a ratio is used of the Bull expressions [2] for the longitudinal and transverse relaxation times for spin 3/2 nuclei undergoing two-site exchange where one site is not under conditions of extreme narrowing, i.e.,

$$\frac{1/T_2 - 1/T_{2f}}{1/T_1 - 1/T_{1f}} = \frac{0.6 + 0.4(1 + 4\omega^2\tau_c^2)^{-1} + (1 + \omega^2\tau_c^2)^{-1}}{1.6(1 + 4\omega^2\tau_c^2)^{-1} + 0.4(1 + \omega^2\tau_c^2)^{-1}} \quad (2)$$

where $\omega = 2\pi$ (4.65×10^6) and for our case $T_{2f} = T_2(l) = 50$ msec and $T_{1f} = T_1(l) = 55$ msec. As shown in Fig. 2 (inset), the value for T_2 is 7.3 msec and T_1, as before, is 15.3 msec. The calculated value of τ_c is 3.89×10^{-8} sec. Following Marshall [10], the inverse of the correlation time is written as the sum of two quantities

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_b}. \quad (3)$$

Since τ_r is the reorientation correlation time for the ion binding site which is channel within the lipid bilayers, this time would be more than an order of magnitude longer than the experimental value for τ_c. Therefore $1/\tau_c \simeq 1/\tau_b$ where τ_b is the occupancy time for the ion in the high concentration binding site. As the inverse of the ion occupancy time is the off-rate constant, this gives $1/\tau_c \simeq 1/\tau_b = k_{\text{off}}^w = 2.6 \times 10^7$/sec.

Thus from the potassium-39 NMR data a binding constant of 8.3/M and an off-rate constant of 2.6×10^7/sec are found for high ion activities.

Discussion

The foregoing results could be utilized in a straightforward manner to calculate single-channel currents if the two dominant states of the channel were the empty channel and the singly occupied channel as has been suggested on the basis of concentration dependence of conductance and of flux data [4, 11]. The physical method data on this channel-lyso-PC system, instead, demonstrate a tight binding process giving rise to single ion occupancy and a weak binding process resulting in double ion occupancy. This has been demonstrated from the viewpoint of the ion using lithium-7 [16], sodium-23 [20, 21] and cesium-133 [15] NMR and from within the channel for potassium and thallium ions using synthetic gramicidin A molecules in which a single carbonyl carbon was enriched [17]. If the off-rate constant for the singly occupied channel were sufficiently small (for example 3×10^5/sec as reported for sodium ion, reference 21) that it did not contribute significantly to the measured ion fluxes, then cy-

cling between singly and doubly occupied states could be occurring with little evidence for the initial ion occupancy. In this case the role of the second ion binding process would be to convert the channel state to one where an ion off-rate constant resulted in a more readily measured current. In the case of sodium ion the second ion binding process increases the off-rate constant for an ion leaving the channel from 3×10^5/sec to 2.3×10^7/sec [20, 21].

In making use of the data reported here, initially, advantage is taken of the "electrically silent" nature of the singly occupied state for sodium ion and it is extended to the potassium ion. Subsequently, reasonable estimates based on NMR data can be used for another two rate constants, and dielectric relaxation results can provide an estimate of the rate constant for the central barrier. In this way the complete calculation can be made as has been previously done for the sodium ion [20, 21].

Taking the entrance-exit barrier to be rate limiting on the positive side of the membrane to which a 100 mV potential is applied, considering only the singly occupied and doubly occupied states with probabilities χ_s and χ_d, respectively, where $\chi_s + \chi_d = 1$, recognizing that $k^w_{on} = K^w_b k^w_{off}$ and using Eyring rate theory to introduce voltage dependence gives for the single-channel current, i,

$$i = \text{forward rate} - \text{backward rate} \tag{4}$$

$$i = [K^+]k^w_{on}\chi_s e^{l_f zFE/2dRT} - k^w_{off}\chi_d e^{-l_b zFE/2dRT} \tag{5}$$

or

$$i = \frac{K^w_b k^w_{off}}{1 + K^w_b}\left(e^{l_f zFE/2dRT} - e^{-l_b zFE/2dRT}\right) \tag{6}$$

where $[K^+]$ is 1 M ion activity.

Equation (6) uses the relationship $\chi_s = (1 + K^w_b)^{-1}$ and $\chi_d = K^w_b(1 + K^w_b)^{-1}$. The exponential term introduces voltage dependence with l_f and l_b being forward and backward distances from binding site to barrier. From the channel structure and binding site locations, $l_f \simeq l_b \simeq 3$ Å; z is the charge on the ion; F is the Faraday, 23 kcal/mole volt; E is the applied potential, 0.1 V; $2d$ is the total distance across the channel, 30 Å; R is the gas constant, 1.987 cal/mole-deg; and T is the temperature in degrees Kelvin. Equation (6) is written for potassium ion at 1 molar activity. This gives

$$i = \frac{8.3}{1 + 8.3} \times 2.6 \times 10^7(e^{0.38} - e^{-0.38})$$
$$= 1.8 \times 10^7 \text{ ions/sec} \tag{7}$$

and

$$\gamma = \frac{i \times 1.6 \times 10^{-19} \text{ coulombs/ion}}{0.1 \text{ volt}}$$
$$= 28.9 \text{ picosiemens (pS)} \tag{8}$$

where γ is the single-channel conductance. Compare this to 25 pS for the mean of the most probable conducting state and to 20 pS for the mean of all states when using diphytanoyl phosphatidylcholine membranes, 0.6 molar activity KCl and 30.7°C [13]. Similar approximations can be made for sodium ion with $K^w_b = 0.6$/M [18] and $k^w_{off} = 2.3 \times 10^7$/sec [20] to give $\gamma(Na) = 10.8$ pS and for rubidium ion with $K^w_b = 2.1$/M and $k^w_{off} = 7.7 \times 10^7$/sec [19] to give $\gamma(Rb) = 58.7$ pS. The resulting conductance ratios would be $\gamma(K)/\gamma(Na) = 2.7$ and $\gamma(Rb)/\gamma(Na) = 5.4$. While these ratios are a little high for most lipids [1, 3, 7, 8], they clearly show the ability of the quadrupolar NMR-derived rate constants to result in reasonable values for the series of three alkali metal ions. As is considered next, a more complete calculation brings the ratios closer to those directly obtained from conductance measurements.

In order to carry out a more complete calculation of the single-channel currents for potassium ion, as had been done with NMR-derived constants for sodium ion [20, 21], values are required for K^t_b, k^t_{off} and k_{cb}. Using carbon-13 NMR, an estimate of $K^t_b \simeq 28$/molar activity has been obtained at 70°C [17]. As the temperature dependence of the tight binding constant for sodium ion has been reported [18], this is used to correct the 70°C tight binding constant for potassium ion to 30°C; a value of 50/M is estimated. Interestingly, the off-rate constants determined at high ion activity are similar for Na^+ (2.3×10^7/sec) and K^+ (2.6×10^7/sec) so that these ions could be expected to have similar off-rate constants for an ion leaving the singly occupied channel. The value used for potassium ion is (2.6/2.3) 3 $\times 10^5$/sec = 3.4×10^5/sec. For k_{cb} a value of greater than 5×10^7/sec has been found for thallium ion in the gramicidin channel (R. Henze and D.W. Urry, *unpublished data*); this value, which is greater than the 4×10^6/sec value found for the malonyl dimer [6], will be used for all three ions. Using the same formalism as applied to the sodium ion [20, 21], $\gamma(K) = 23.6$ pS. For rubidium ion the tight binding constant is taken to be the same as for the cesium ion (5), i.e., $K^t_b = 60$/M, and k^t_{off} is evaluated as the ratio (7.7/2.3) times 3×10^5/sec to be 10^6/sec. This yields $\gamma(Rb) \simeq 51$ pS. For the sodium ion the best values obtained by NMR are $K^t_b \simeq 30$/molar activ-

ity, $K_b^w = 0.6$/molar activity [18], $k_{off}^t = 3 \times 10^5$/sec and $k_{off}^w = 2.3 \times 10^7$/sec [20, 21] and $k_{cb} = 5 \times 10^7$/sec. At 1 molar activity, γ(Na) becomes 11.8 pS. The conductance ratios calculated using NMR-derived constants and the dielectric relaxation result for the central barrier become $\gamma(K)/\gamma(Na) = 2.0$ and $\gamma(Rb)/\gamma(Na) = 4.3$; these ratios are remarkably close to literature values, e.g., 1.7 to 2.6 for the former and 2.9 or greater for the latter [1, 3, 7, 8].

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