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Biochimica et Biophysica Acta 1238 (1995) 12–21

BBA Biochimica et Biophysica Acta

Ion pair binding of Ca^{2+} and Cl^- ions in micellar-packaged gramicidin A

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Received 22 September 1994; revised 27 March 1995; accepted 30 March 1995

Abstract

The two independent NMR experiments were performed to investigate the interaction between $CaCl_2$ and the gramicidin A (GA) ion transport channel, using ^{13}C-enriched GA and GA molecules incorporated into dodecylphosphocholine (DPC) micelles. The chemical shifts of C-13 labeled carbonyl carbons vs. $CaCl_2$ concentration demonstrate that Ca^{2+} and Cl^- ions interact as an ion pair within the GA structure with the Cl^- ion located near the position of the carbonyl group of the Trp^{11} residue some 5.5 Å from the mouth of the GA helix, and the Ca^{2+} ion bound at the position of the carbonyl group of the Trp^{15} residue some 2.5 Å from the entrance to the helical pore. The measurements of the ^{35}Cl line-widths and transverse relaxation times illustrate that the interaction occurs between Cl^- ions and GA in DPC when in $CaCl_2$ solution, that no interaction is detected between Cl^- ions and GA in DPC when in NaCl solution, and that the interaction between Cl^- ions and GA in DPC when in $MgCl_2$ solution is much weaker than in $CaCl_2$ solution. In short, a Cl^- ion can enter the GA when it is paired with a divalent Ca^{2+} ion; and Ca^{2+} and Cl^- ions as a pair exchange rapidly with sites of the GA dimer.

Keywords: NMR, ^{13}C-; NMR, ^{35}Cl-; Transverse relaxation time; Calcium chloride titration; Dodecylphosphocholine (DPC) micelle; Circular dichroism

1. Introduction

Gramicidin A (GA), a linear pentadecapeptide, 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}-$NHCH_2CH_2OH$, produced by *Bacillus brevis*, forms a transmembrane ion-channel [1,2]. A structure of the GA membrane-spanning channel was originally proposed to be single-stranded $\beta^{6.3}$-helices, head-to-head (N-terminus to N-terminus) dimerized by six hydrogen bonds with the helices in the left-handed helical sense by Urry and co-workers [3,4], and a structure was identified by two-dimensional NMR in negatively charged sodium dodecyl sulfate (SDS) micelles [5] and in neutral dodecylphosphocholine (DPC) micelles [6] to be the same as the structure previously described in lysophosphatidylcholine (LPC) vesicles by Urry and co-workers [7,8] except that in the micelles the structure is right-handed.

The GA transmembrane channel as a model for the biological channels has received much attention; however, there are still areas of disagreement, such as the molecular mechanism of ion selectivity by the GA channel. It was shown early that the GA channel is essentially impermeable to calcium ions and chloride ions [9]. But $CaCl_2$ does affect the magnitude of the single-channel current of monovalent cations and does change the current–voltage characteristic from an almost linear to a strong saturating behavior. These findings were interpreted to mean that the GA channel conductance was affected by the interaction of a divalent ion at or near its entrance [10]. The Ca^{2+} ion binding sites were located at the position of the Trp^{15} residue of the peptide channel, using the ^{13}C-enriched carbonyl carbon gramicidin A incorporated into LPC vesicles. The hypothesis whereby the divalent ion was presented from crossing through the GA channel was the repulsive image force; this force is proportional to the square of ion charge [11,12]. According to the dependence of the spin-lattice relaxation rates of the GA protons on Mn^{2+} ion concentration, it was inferred that Mn^{2+} ions were bound at the channel mouths about 6.4, 8.6, and 8.8 Å (±2 Å) away from the carbonyl oxygen atoms of D-$Leu^{12,14,10}$ residues, using GA molecules incorporated into SDS detergent micelles [13]. In this case, the argument concerning the impermeability of the divalent cations in the GA channel was that the size of the divalent cation,

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SSDI 0005-2736(95)00094-1

retaining its hydrated shell, was too large to enter the pore. The effect of the negatively charged surface of SDS detergent micelles on the ion interactions of the GA channel in SDS micelles has yet to be properly evaluated.

In the present study, titrations were carried out using ^{13}C-NMR and ^{35}Cl-NMR methods with gramicidin A and ^{13}C-labeled gramicidin A incorporated into DPC micelles. By comparison with the results of the NaCl titration of the GA channel in DPC micelles, the interaction between $CaCl_2$ and the peptide channel has been demonstrated by ^{13}C chemical shifts and by ^{35}Cl line-width and ^{35}Cl transverse relaxation studies as a function of $CaCl_2$ concentration. Based on the conformation of the GA channel in DPC micelles as a right-handed, single-stranded, β-helical dimer [6], the binding sites and the binding constants of Ca^{2+} and Cl^- ions were evaluated by analysis of the ^{13}C- and ^{35}Cl-NMR data.

2. Materials and methods

The samples of enriched [1-^{13}C]Trp^{11} GA, [1-^{13}C]Trp^{13} GA, and [1-^{13}C]Trp^{15} GA for ^{13}C-NMR study were synthesized and verified in this laboratory, as previously described [11,14]. The gramicidin samples for the ^{35}Cl-NMR study were purchased from Nutritional Biochemicals Corporation (Cleveland, OH) as a mixture of gramicidin A, B, and C, then purified by gel filtration over a Sephadex LH-20 column using methanol for elution. The dodecyl phosphocholine, purchased from Avanti Polar-Lipid (Birmingham, AL), was employed to form lipid micelles. All chemicals used were of optical grade.

2.1. Sample handling

The appropriate amounts of gramicidin A (^{13}C-enriched gramicidin A or [Val-1] gramicidin A) (0.006 M) and dodecyl phosphocholine (DPC) (0.2 M) were mixed in 1 ml of 2,2,2-trifluoroethyl alcohol (TFE) at 25°C for 2 h. Then TFE was removed, and 0.7 ml of water (D_2O, 99.83%) was added to the samples. After being sonicated, the samples were incubated in a water bath at 70°C for 3 days. Next, the samples were centrifuged, and the supernatant was extracted for the NMR study. The conformation of the gramicidin molecules incorporated into DPC micelles was assessed by circular dichroism (CD), and the concentration of the GA channel in the supernatant was determined by absorbance measurements, using a molar extinction coefficient 45000 cm^{-1} M^{-1} in methanol at 280 nm, as previously described [7].

2.2. NMR measurements

The ^{13}C-NMR spectra were obtained at 25 MHz and 25 ± 1°C, using 8 k data points, 20 μs 90° pulse, a delay time of 2 s, and a 5000 Hz spectral width. An input power of 15 W was applied continuously for broad-band decoupling of protons. The ^{35}Cl-NMR spectra were performed at 9.7 MHz at room temperature (25 ± 1°C). The experimental conditions were carried out with 30 μs pulse widths for 90° and 2 s for an interval time. The transverse relaxation times were measured by the spin-echo method using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence ($90° \sim (\sim \tau \sim 180° \sim \tau)_n$). The data were collected from 14 different loop numbers to determine the decay of the signal. The transverse relaxation time T_{2f} of 100 mM $CaCl_2$ in D_2O, 28 ms, was used as reference for free Cl^- ions.

3. Results and discussion

3.1. The structure verification

The CD spectra (Fig. 1) of the GA molecules incorporated into DPC micelles in D_2O without $CaCl_2$ (solid line) and with 2 M $CaCl_2$ (dashed line) indicate that the conformation of the GA molecules in DPC micelles is the same as that of malonyl GA molecules in DPC micelles [6]; this conformation was identified to be a right-handed, head-to-head, single-stranded, $\beta^{6.3}$-helical dimer by NOE spatial connectivities. The CD spectra also suggest that the conformation of gramicidin A in DPC micelles fully saturated with $CaCl_2$ is identical to that in the ion-free state. Accordingly, no conformational change was considered when calcium chloride ions interacted with gramicidin A in DPC micelles.

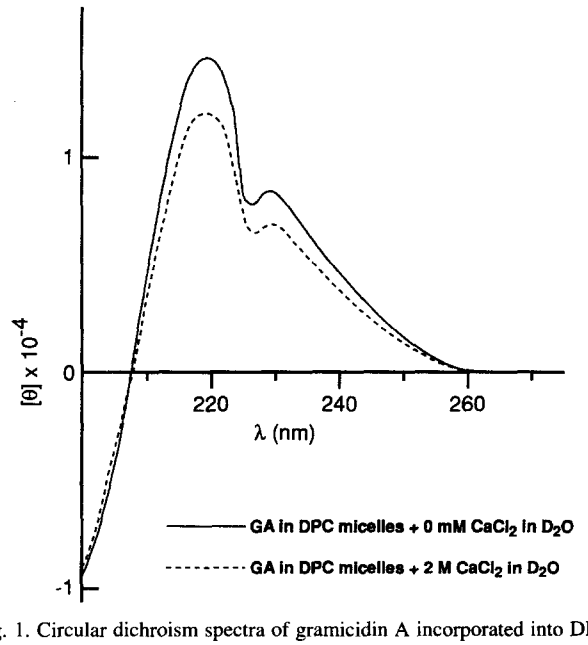

Fig. 1. Circular dichroism spectra of gramicidin A incorporated into DPC micelles in D_2O without $CaCl_2$ (A, solid line) and with 2 M $CaCl_2$ (B, dashed line) that were recorded by a Cary 60 spectropolarimeter at room temperature using a 0.1 mm optical length. The spectra show the same conformation of gramicidin A in DPC micelles in the absence of and in the presence of the calcium chloride ions.

3.2. ^{13}C chemical shifts induced by $CaCl_2$

It has been shown that the ion-induced chemical shift of carbonyl carbons on ^{13}C-NMR is a sound method with which to determine the carbonyls involved in ion binding [15] and to locate the binding sites of the GA channels, using C-13 labeled gramicidin A incorporated into lipid vesicles [11,14]. The same method was also employed here to study the interaction between $CaCl_2$ and the GA channels, using ^{13}C-labeled gramicidin A incorporated into DPC micelles.

In Fig. 2A, the two ^{13}C-NMR spectra are overlapped:

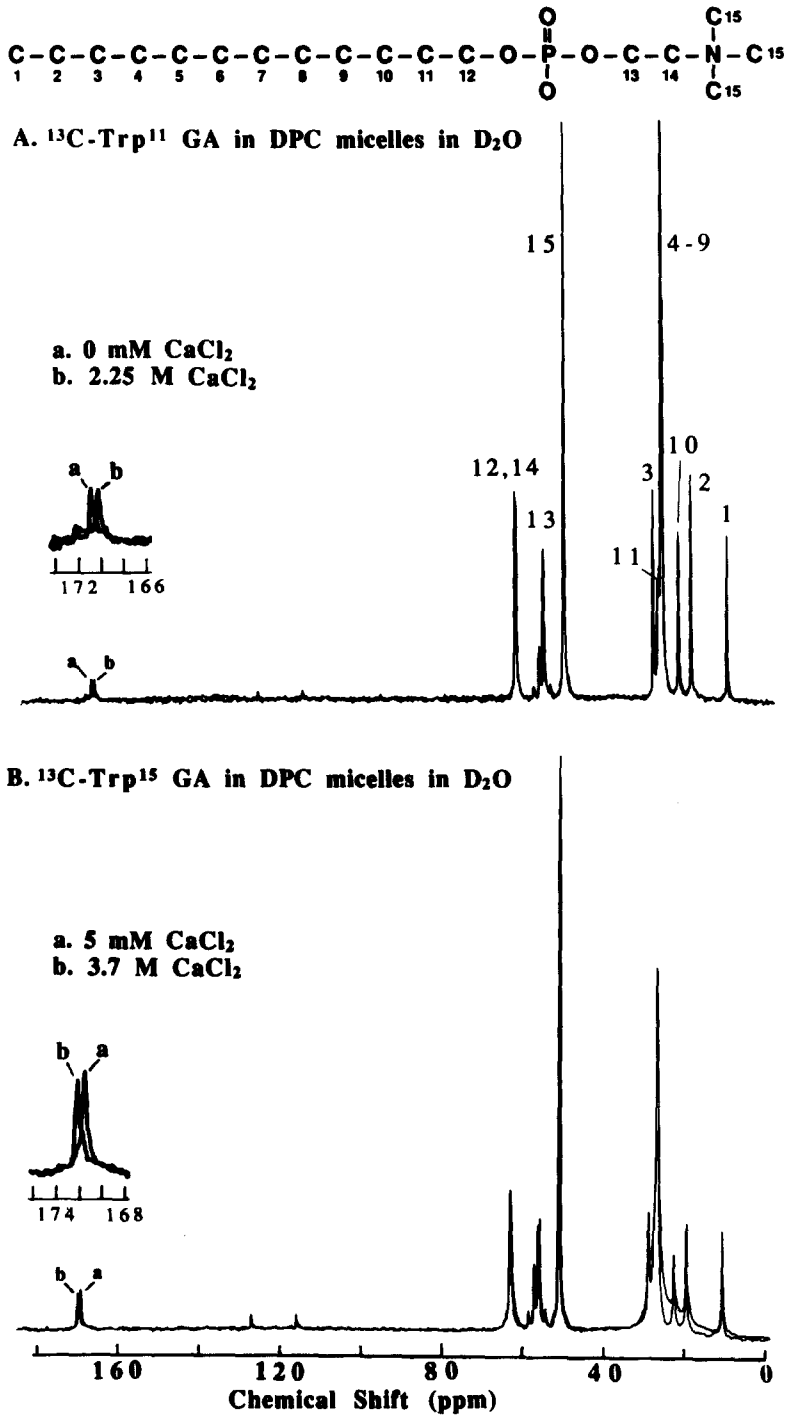

Fig. 2. ^{13}C-NMR spectra obtained at 25 MHz and 25°C. (A) The two overlapped spectra of [1-^{13}C]Trp^{11} gramicidin A incorporated into DPC micelles in the presence of 0 mM (a) and 2.25 M (b) $CaCl_2$. The signal of the carbonyl carbon of Trp^{11} residue shifts 0.71 ppm to a higher field from 0 mM to 2.25 M $CaCl_2$. (B) The two overlapped spectra of [1-^{13}C]Trp^{15} gramicidin A in DPC micelles in 5 mM (a) and 3.7 M (b) $CaCl_2$. The signal of the carbonyl carbon of Trp^{15} residue shifts 0.64 ppm to a lower field from 5 mM to 3.7 M $CaCl_2$. All of the signals of the DPC micelles exhibit no detectable shift in both spectra A and B.

one is the spectrum of the ^{13}C-enriched carbonyl carbon [1-^{13}C]Trp^{11} gramicidin A without $CaCl_2$ (a); the other is the spectrum of the same sample in the present of 2.25 M $CaCl_2$ (b). All of the resonance signals of the spectra are assigned. The small signal in the low field is from the 90% ^{13}C-enriched carbonyl carbon of gramicidin A, and the lower intensity of the signal is due to the ratio of gramicidin A to lipid (1:33). The spectra show that the signal of the carbonyl carbon of gramicidin A in the Trp^{11} position shifted 0.71 ppm to a higher field (or a lower frequency), called negative shift, while the signals of DPC micelles exhibited no shift when the concentration $CaCl_2$ was increased from zero to 2.25 M. In Fig. 2B, to the contrary, the signal of the carbonyl carbon of [1-^{13}C]Trp^{15} gramicidin A shifted 0.64 ppm to a lower field (or a higher frequency), called positive shift, on increasing the concentration of $CaCl_2$ from 5 mM to 3.7 M. The $CaCl_2$ titration study was carried out by ^{13}C-NMR experiments, using ^{13}C-enriched carbonyl carbons of [1-^{13}C]Trp^{11,13,15} gramicidin A molecules in DPC micelles. The chemical shifts of the C-13 labeled carbonyl carbons were measured by reference to the signal of the fatty acid chain, C^4-C^9, at 29.10 ppm since the resonance signals of DPC micelles did not shift with the $CaCl_2$ concentration. The chemical shifts ($\Delta\delta$) were plotted as a function of the concentration of $CaCl_2$ (Fig. 3).

Important information was obtained from this plot: (1) the carbonyl carbon of [1-^{13}C]Trp^{11} gramicidin A shifted to a higher field as the $CaCl_2$ concentration was increased; this negative shift has never been observed before in the monovalent cation titrations [8,14], nor in the $CaCl_2$ titrations using C^{13}-labeled tryptophan gramicidin A incorporated into LPC vesicles [11]. (2) The carbonyl carbon of [1-^{13}C]Trp^{13} gramicidin A has a small, almost negligible negative shift (−0.07 ppm), on increasing the $CaCl_2$ concentration. This is very different with the monovalent cation titrations, in which the carbonyl carbon of [1-^{13}C]Trp^{13} gramicidin A exhibits a large positive shift as a function of the ion concentration [8,14]. (3) As the $CaCl_2$ concentration is raised, the carbonyl carbons of [1-^{13}C]Trp^{15} gramicidin A and [1-^{13}C]Trp^{11} gramicidin A have symmetric chemical shifts in opposite direction, compared with the chemical shifts of carbonyl carbon of [1-^{13}C]Trp^{13} gramicidin A.

According to the right-handed structure of gramicidin A in DPC micelles, the positions of the carbonyl carbons of Trp^{11,13,15} residues are buried in the DPC micelles and the distances of the carbonyl oxygen atoms of Trp^{11,13,15} residues to the mid-point of the channel are about 7.1, 8.6, and 10.2 Å based on the repeating distance of 1.51 Å/dipeptide [16]. The carbonyl oxygens of the peptide residues will liberate into the channel to form ion-peptide interactions of the type N-C = O · · · Ca^{2+} (or Cl^-) at the binding site when ions enter the single-file channel; this is in agreement with the experimental observations on solid-state NMR [17,18] and the molecular dynamic computations [19,20]. Therefore, the chemical shifts of the C^{13}-enriched carbonyl carbons are induced by the interaction between ions and the peptide at the binding sites.

From the NMR chemical shift concept, if the electron density around the magnetic nucleus is reduced, the deshielding leads to a lower field shift (or a higher frequency shift); otherwise, increasing the electron density will result in a higher field shift (or a lower frequency shift). In Fig. 3, it seems clear that the chemical shift of the carbonyl carbon in Trp^{11} position is induced by a negatively charged ion, and the chemical shift of the carbonyl carbon in Trp^{15} position is induced by a positively charged

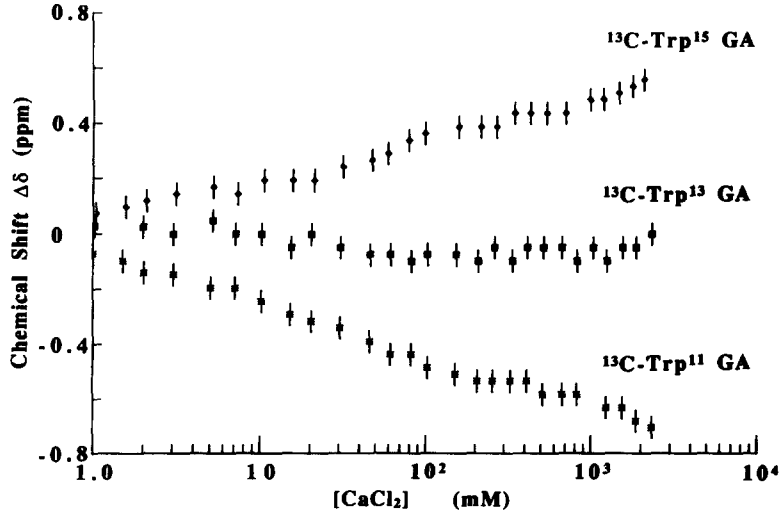

Fig. 3. The chemical shift, $\Delta\delta$ (ppm), of ^{13}C-enriched carbonyl carbons of Trp^{11,13,15} gramicidin A induced as a function of $CaCl_2$ concentration. The resonance of the carbonyl carbon of the Trp^{11} residue shifts to a higher field and that of the Trp^{15} residue shifts to a lower field when $CaCl_2$ concentration is increased. They have symmetric chemical shifts in opposite directions. The resonance of the carbonyl carbon of the Trp^{13} residue almost remains unshifted as the $CaCl_2$ concentration increases (see text for details).

ion since the electron density of the carbonyl carbon (C = O) would be increased by an anion and decreased by a cation. The explanation in this case is that Ca^{2+} and Cl^- ions enter the GA channel as a pair, the Ca^{2+} ion is bound at the Trp^{15} position, but the Cl^- ion is bound at the Trp^{11} residue so that the carbonyl carbons of [1-^{13}C]Trp^{11} and [1-^{13}C]Trp^{15} gramicidin A have mirrored chemical shifts. The carbonyl carbon of the Trp^{13} residue is in a neutral position, and the small negative shift could be affected by the Cl^- ion due to its larger size.

3.3. Analysis of the ^{13}C chemical shifts

The data analysis employed to determine the binding constants of the GA channels for Ca^{2+} and Cl^- ions is based on the two equations which have been used before [21,22]. The equation for double occupancy is given by

$$\Delta\delta = \frac{C_1}{C_T}\Delta\delta_t + \frac{C_2}{C_T}\Delta\delta_w \quad (1)$$

where C_1 and C_2, respectively, are the concentrations of singly and doubly occupied channel molecules; C_T is the concentration of the total channel molecules, and $\Delta\delta_t$ and $\Delta\delta_w$ are the chemical shifts affected by the singly and doubly occupied states. For the binding constant for the singly occupied state, $K_b^t = C_1/(2[X]C_0)$ and for the binding constant for the doubly occupied state, $K_b^w = 2C_2/([X]C_1)$ where $[X]$ is the concentration of free ions and C_0 is the concentration of the empty channel molecules. For single occupancy, the equation is given by

$$\Delta\delta = \frac{C_1}{C_T}\Delta\delta_b \quad (2)$$

where δ_b is the maximal ^{13}C chemical shift for each sample, and for the binding constant, $K_b = C_1/([X]C_0)$ where $C_0 = C_T - C_1$; and $[X] = [X]_T - C_1$ ($[X]_T$ is the total ion concentration). After many calculations using estimated constants, the best fitting values were searched and the curves were plotted by computer as shown in Fig. 4.

From this analysis, it is found that the analytical curves for double occupancy fit well for both the chemical shift data for [1-^{13}C]Trp^{11} and for [1-^{13}C]Trp^{15} gramicidin A. The average deviations between the best fitted curves for double occupancy and the chemical shifts of the carbonyl carbons of Trp^{11} and Trp^{15} residues are 4.5% and 5.7%; however, the average deviations between the best fitted curves for single occupancy and the data for Trp^{11} and Trp^{15} residues are 15.7% and 16.4%. Consequently, double occupancy is considered relevant to the interaction between $CaCl^2$ and gramicidin A in DPC micelles. It is also found that the binding constants, K_b^k and K_b^w, obtained from the analysis of the chemical shifts of [1-^{13}C]Trp^{11} gramicidin A are 120/M and 6/M for Cl^- ions, and from the analysis of the chemical shifts of [1-^{13}C]Trp^{15} gramicidin A are 180/M and 6/M for Ca^{2+} ions since the chemical shifts of [1-^{13}C]Trp^{11} gramicidin A in DPC micelles was considered to be induced by Cl^- ions and that of [1-^{13}C]Trp^{15} gramicidin A was taken as due to Ca^{2+} ions. The two binding constants for the doubly occupied state are an identical 6/M. And the two binding constants for the singly occupied state, 120/M and 180/M, are also similar, that is, the two dissociation constants for the singly occupied state, $1/K_b^t$ are very close: 8.3 mM for [1-^{13}C]Trp^{11} gramicidin A and 5.6 mM for [1-^{13}C]Trp^{15} gramicidin A. Therefore, the binding constants, K_b^t and

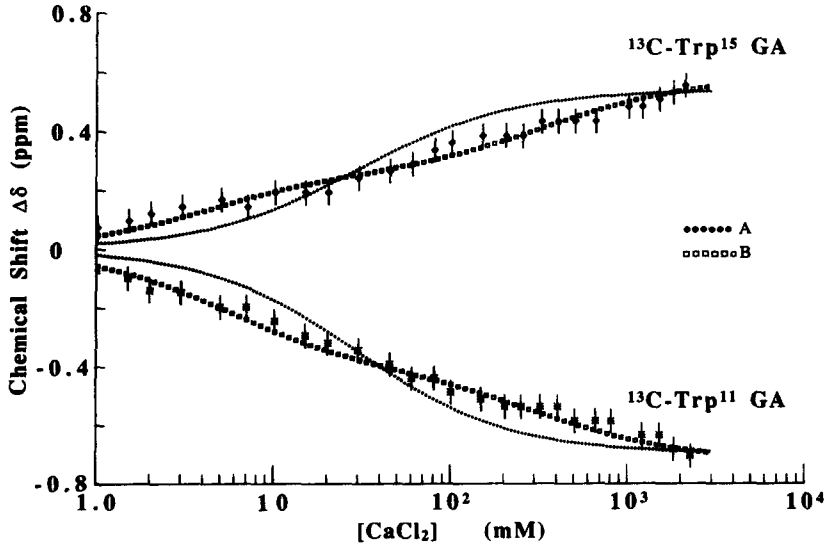

Fig. 4. The data analyses of the ^{13}C chemical shifts of the carbonyl carbons of Trp^{11} and Trp^{15} gramicidin A vs. $CaCl_2$ concentration: one for single occupancy (A) and one for double occupancy (B). The average deviations between the $\Delta\delta$ of [1-^{13}C]Trp^{11} and [1-^{13}C]Trp^{15} gramicidin A and the analytical curves of the double occupancy are 4.5% and 5.7%; and these between the $\Delta\delta$ of [1-^{13}C]Trp^{11} and [1-^{13}C]Trp^{15} gramicidin A and the analytical curves of the single occupancy are 15.7% and 16.4%.

K_b^w, for Cl^- ions are the same as those for Ca^{2+} ions; this is consistent with the contention that Ca^{2+} and Cl^- ions enter the GA channel as an ion pair. This perspective can be tested by directly observing the Cl^- interaction by NMR relaxation methods.

3.4. Measurement of ^{35}Cl line-width

The observable NMR relaxation rates or line-widths are determined by the rate at which the nuclei exchange between different environments, by the distribution of the nuclei among the different environments, and by the intrinsic relaxation rates at each environment. From the relaxation rates, the information on field gradients and molecular mobility at the binding sites can be obtained. Using ^{35}Cl as a probe, the interaction between the anion and the GA channel can be observed and the binding constants for both the singly and doubly occupied states can be estimated by means of ^{35}Cl-NMR. The ^{35}Cl-NMR study of the line-width as a function of Cl^- concentration utilized four titrations (Fig. 5): (A) the $CaCl_2$ titration of free DPC lipid micelles in D_2O; (B) the NaCl titration of gramicidin A incorporated into DPC micelles in D_2O; (C) the $MgCl_2$ titration of gramicidin A incorporated into DPC micelles in D_2O; and (D) the $CaCl_2$ titration of the gramicidin A incorporated into DPC micelles in D_2O. The concentrations of the GA channels of the samples for B, C, and D were confirmed to be an essentially identical 2 mM by absorbance at 280 nm. The titrations were run from 10 mM to 1.2 M with 16 data points.

The line-width was measured at half-height of the NMR absorption curve and the line-width is related to the transverse relaxation time, T_2^*, as $\Delta\nu = 1/\pi T_2$. Plotting the ^{35}Cl line-width $\Delta\nu$ vs. Cl^- ion concentration shows that the line-width of Cl^- ions in DPC micelles without gramicidin A (A) remains about 17 Hz on increasing the $CaCl_2$ concentration, which was taken as a reference value. In the NaCl titration, the ^{35}Cl line-width observed for gramicidin A in DPC micelle (B) is also narrow (22.6 Hz) and also remains unchanged as the NaCl concentration is changed. Compared with the reference, the narrow and stable line-width in (B) is due to the Cl^- ions in the free state, with no influence from the Cl^- ions of a bound state. This is in agreement with the earlier study: no single-channel current due to Cl^- ions was observed from the GA channel in planar lipid bilayers bathed in a NaCl solution [9]. It is also consistent with the ^{13}C chemical shifts on ^{13}C-NMR using C^{13}-labeled gramicidin A incorporated in DPC micelles; there is no carbonyl carbon chemical shift induced by Cl^- ions in NaCl solution. However, in the $CaCl_2$ titration, the ^{35}Cl line-width obtained for gramicidin A in DPC micelles (D) is much broader than that for DPC micelles without gramicidin A (A), and broader than that in the NaCl titration (B).

The broader line-width in (D) decreases as $CaCl_2$ concentration is increased. The relaxation time of the quadrupolar nuclei depends on the magnitude of the quadrupolar coupling constant ($(e^2qQ)/\hbar$) and the modulation of the correlation time (τ_c). As the values of the spin quantum number, I, and the quadrupolar moment, eQ, are

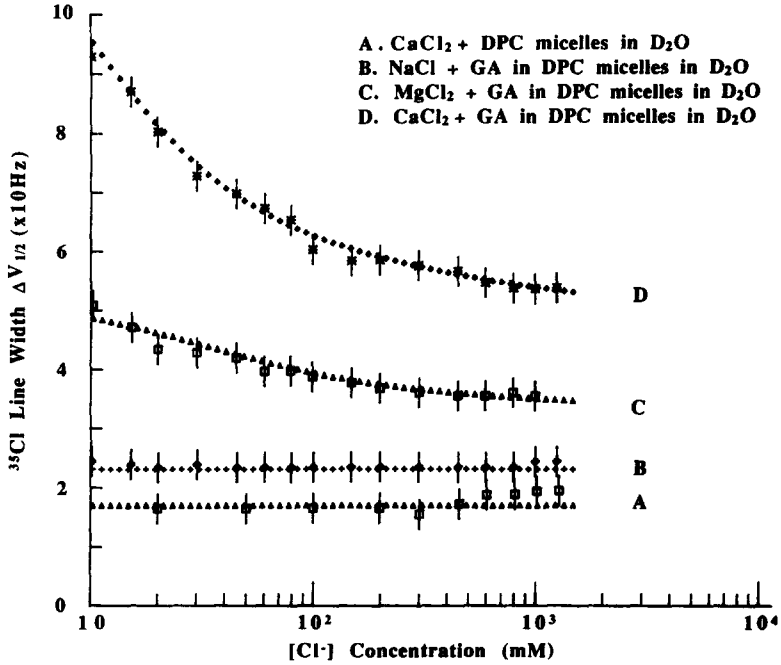

Fig. 5. The ^{35}Cl line-widths of the four samples as a function of the Cl concentration: (A) the $CaCl_2$ titration of the free DPC micelles in D_2O; (B) the NaCl titration of gramicidin A in DPC micelles in D_2O; (C) the $MgCl_2$ titration of gramicidin A in DPC micelles in D_2O; and (D) the $CaCl_2$ titration of gramicidin A in DPC micelles in D_2O. The curves fitting the ^{35}Cl line-width data are plotted using the calculations of the line-width analysis (see text for details).

constants for the ^{35}Cl nuclei, the τ_c of the Cl^- ions bound by the peptide channels is increased due to the slow-tumbling of the GA channels. The interaction between the Cl^- ions and the peptide channels and the interaction between the Cl^- and Ca^{2+} ion pairs in the GA channels would result in a larger value of the electric field gradient, *eq*, for the bound Cl^- ions (see next section). The increases in τ_c and *eq* will cause a broader line-width or a shorter relaxation time as given by

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{3(2I+3)}{40I^2(2I-1)}\left(\frac{1+\eta^2}{3}\right)\left(\frac{e^2qQ}{\hbar}\right)\tau_c \qquad (3)$$

3.5. Analysis of the ^{35}Cl line-widths

Based on the ^{13}C chemical shift analysis, the double occupancy model is relevant for analysis of the line-widths. Usually the observed line-width is composed of the three partial line-widths in the different states:

$$\Delta\nu_{obs} = P_f\Delta\nu_f + P_t\Delta\nu_t + P_w\Delta\nu_w \qquad (4)$$

where $\Delta\nu_f$ is the line-width of Cl^- ions in the free state; $\Delta\nu_t$ and $\Delta\nu_w$ are the line-widths of Cl^- ions for the singly and doubly occupied states; and P_f, P_t, and P_w are the probabilities of the Cl^- ions occurring in the free, singly-occupied, and doubly-occupied channel states. The probability of the ions in the free state, P_f, is equal to $[X]/[X]_T$ where $[X]$ and $[X]_T$ are the concentrations of the free ions and the total ions. Since the total GA channel concentrations of the sample B, C, and D were identified as 2 mM and the titrations were carried out from 10 mM to 1.2 M, the values of P_f $(=[X]/[X]_T)$ can be taken as one. Accordingly, Eq. (4) can be written as

$$\Delta\nu_{obs} - \Delta\nu_f = P_t\Delta\nu_t + P_w\Delta\nu_w \qquad (5)$$

It may be noted for the double occupancy model in the absence of an applied field that there are three occupied states in the GA channels: oo, xo (or ox), and xx [11]. The binding constant for the singly occupied state, K_b^t, is involved in oo ⇔ xo or oo ⇔ ox while the binding constant at the doubly occupied state, K_b^w, is related to xo ⇔ xx or ox ⇔ xx. According to this model, $C_1 = 2k_b^t[X]C_0$, $C_2 = 1/2(K_b^w[X]C_1)$, $C_T = C_0 + C_1 + C_2$, and $[X]_T = [X] + C_1 + 2C_2$, where C_0, C_1 and C_2, respectively, are the concentrations of empty, singly-occupied, and doubly-occupied channel molecules, and C_T is the concentration of the total channel molecules, as previously described [21]. Thus, $P_t = C_1/[X]_T$, $P_w = 2C_2/[X]_T$ and Eq. (5) can be written as

$$\Delta\nu_{obs} - \Delta\nu_f = \frac{C_1}{[X]_T}\Delta\nu_t + \frac{2C_2}{[X]_T}\Delta\nu_w \qquad (6)$$

When the ion concentration is very high, the $C_1/[X]_T \approx 2C_2/[X]_T \approx 0$ then normally $\Delta\nu_{obs} = \Delta\nu_f$, but in the present case, as seen in Fig. 5 and discussed below, even though the concentration is very high such that one would expect $\Delta\nu_{obs} = \Delta\nu_f$, there is an additional line broadening, defined as $\Delta\nu_{pair}$.

In Fig. 5, however, it is found that at the high ion concentration of 1.2 M, the $\Delta\nu_{obs}$ for titration D is still larger than that for the titration B by about 30 Hz, i.e., $\Delta\nu_{obs} > \Delta\nu_f$, because the line-width for titration B is only affected by Cl^- ions in the free state. The analysis of the ^{13}C chemical shifts suggests that the Cl^- and Ca^{2+} ions interact with the GA channel as an ion pair for the $CaCl_2$ solution; hence, the difference between the line-widths for titration B and D at high concentration was considered to be caused by ion pairing. Since charges contribute to the electric field gradient, *eq*, with an r^{-3}, distance dependence and the ion pair charges occur at very short distances, this will have a large influence on the line-width or the relaxation time. Therefore, the equation for fitting the ^{35}Cl line-width for gramicidin A in DPC micelles in $CaCl_2$ solution would be

$$\Delta\nu_{obs} - \Delta\nu_f - \Delta\nu_{pair} = \frac{C_1}{[X]_T}\Delta\nu_t + \frac{2C_2}{[X]_T}\Delta\nu_w \qquad (7)$$

where $\Delta\nu_{pair}$ is the increase in line-width due to the Cl^- and Ca^{2+} ion pair in the peptide channels.

After setting $\Delta\nu_f$ and $\Delta\nu_{pair}$ properly, the best theoretical fit to the ^{35}Cl line-width was searched and plotted by inputting the estimated constants, K_b^t, K_b^w, $\Delta\nu_t$, and $\Delta\nu_w$. The binding constants, K_b^t and K_b^w, for gramicidin A in DPC micelles in the $CaCl_2$ titration were evaluated from this analysis to be 120/M and 4/M for Cl^- ion. The average error between the fitted curve and the experimental data is less than 3%. A similar number, 140/M, is obtained when plotting the reciprocal of the excess linewidth using the high concentration linewidth as the reference and taking the negative *x*-axis intercept as the reciprocal of the binding constant. The binding constants, K_b^t and K_b^w, are essentially identical to those determined by the analysis of ^{13}C chemical shifts on ^{13}C-NMR. Clearly, the analyses of ^{35}Cl line-width also demonstrate that there is an interaction between Cl^- ions and gramicidin A in DPC micelles when in the presence of calcium ion. The fitting of the ^{35}Cl line-width for gramicidin A in DPC micelles for the NaCl titration is a straight line and the two binding constants, K_b^t and K_b^w, are both smaller than 10^{-4}/M. This is consistent with the view that there is no interaction between Cl^- ions and gramicidin A in DPC micelles when in NaCl solution.

In the $MgCl_2$ titration (Fig. 5C), the ^{35}Cl line-width of gramicidin A in DPC micelles in $MgCl_2$ is narrower than in $CaCl_2$, but broader than in NaCl; and the ^{35}Cl line-width decreases when the concentration of $MgCl_2$ is increased. The binding constants, K_b^t and K_b^w for gramicidin A in DPC micelles in $MgCl_2$ were determined by the line-width analysis to be 18/M and 1/M for Cl^- ions. Comparing with the line-widths for titrations B and D, the interaction

between Cl^- ions and gramicidin A in DPC micelles also occurs in $MgCl_2$, but this interaction is much weaker than that in $CaCl_2$. This is in agreement with the single-channel current observations [10]: the effect of blocking the action on the GA channel by adding $MgCl_2$ is much weaker than by adding $CaCl_2$. Therefore, the interaction between Cl^- ions and the GA channel in the presence of magnesium ions is also part of the reason for the blocking.

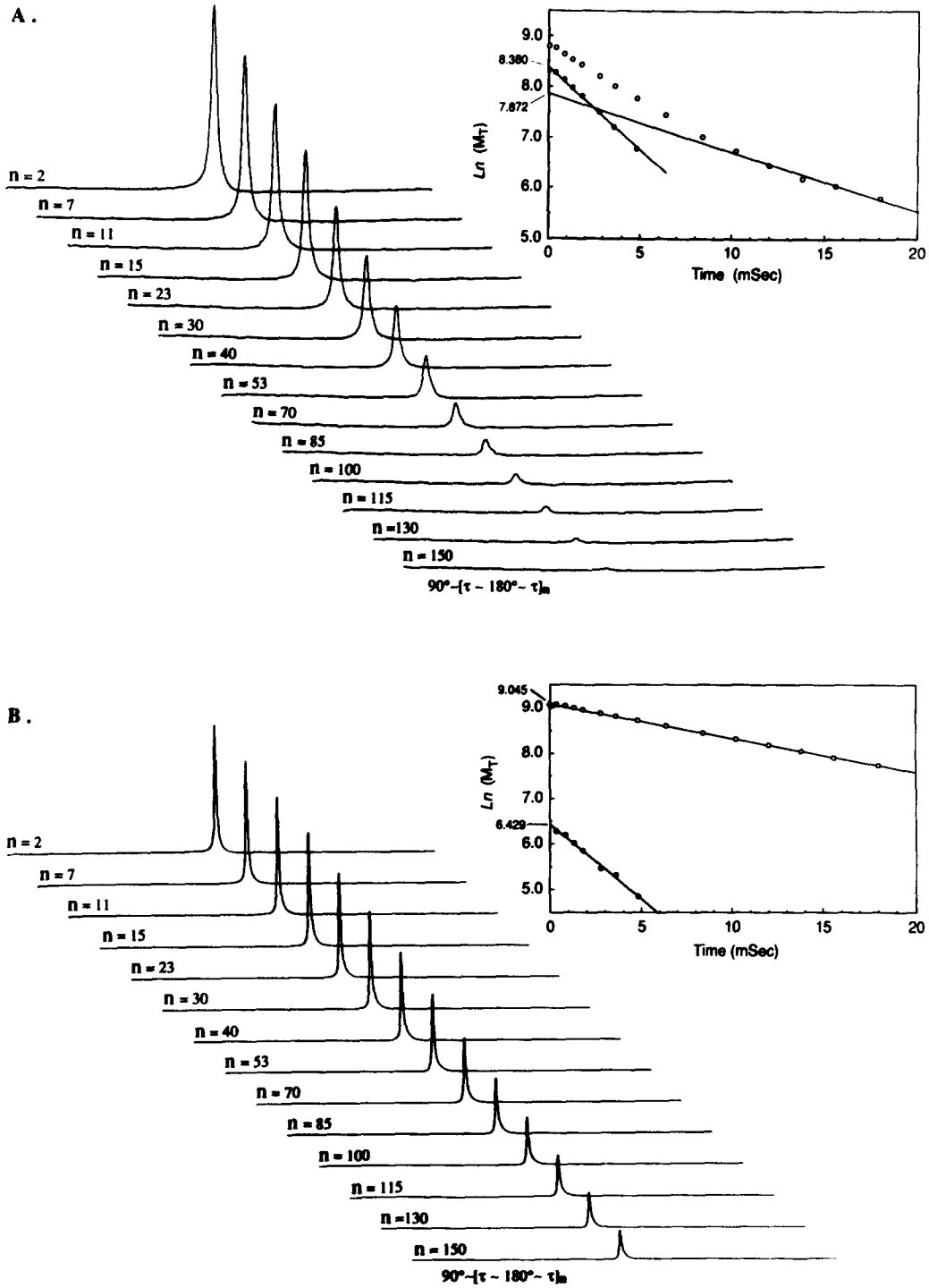

Fig. 6. The ^{35}Cl transverse relaxation times measured by the spin-echo method using the CPMG pulse sequence, a 2 mM channel concentration of gramicidin A in DPC micelles in D_2O and a 2 M ion concentration. In 2 M $CaCl_2$ (A), the insert shows that the ^{35}Cl transverse magnetization is composed of the broad and narrow components, and the ratio of the broad component to the narrow component is determined from exp(8.380)/exp(7.872) as 1.66 (62%:38%). In 2 M NaCl (B), the insert shows that the ^{35}Cl transverse magnetization is essentially a single and narrow component since the broad component is only 6% of the narrow component given by exp(6.259)/exp(9.045). The two experiments were carried out with the same parameters and with the same conditions.

3.6. ^{35}Cl transverse relaxation time study

The ^{23}Na transverse relaxation times (T_2) in the presence of GA molecules in LPC vesicles have been obtained by means of the CPMG pulse sequence [23–26]. Crucial information has been found in those studies. (1) The transverse magnetization of ^{23}Na 3/2-nuclear spin was composed of a broad (or fast) component and a narrow (or slow) component given by

$$M_t = M_0\left(0.6\exp\left(\frac{-t}{T_2'}\right) + 0.4\exp\left(\frac{-t}{T_2''}\right)\right)$$

when a 3 mM concentration of GA channels is present in LPC vesicles. The ratio of the broad component to the narrow component is about 1.5 (60% to 40%). (2) A narrow and single-component decay for ^{23}Na transverse magnetization was observed in LPC vesicle in the absence of GA channels as though all Na^{2+} ions were in free state and $\omega^2\tau_c^2 \ll 1$. (3) Using the fast and slow transverse relaxation times, T_2' and T_2'', the ^{23}Na ion correlation time, τ_c, was calculated by the expression due to [26]

$$\frac{\frac{1}{T_2'} - \frac{1}{T_{2f}}}{\frac{1}{T_2''} - \frac{1}{T_{2f}}} = \frac{1 + \frac{1}{(1+\omega^2\tau_c^2)}}{\frac{1}{(1+4\omega^2\tau_c^2)} + \frac{1}{(1+\omega^2\tau_c^2)}}$$

where T_{2f} is the transverse relaxation time of free Na^+ ions. The reciprocal of the correlation time, τ_c, was deduced to be the off-rate constant, k_{off}^w, for ions leaving the GA channels from the doubly occupied state.

Drawing from the previous studies [25,27], the ^{35}Cl transverse relaxation times (T_2) were measured by the same methods to identify the interaction between Cl^- ions and the GA channels in $CaCl_2$ solutions and in NaCl solutions. Each ^{35}Cl-NMR experiment was performed with the same parameters at the same conditions, using 2 mM concentration of GA channels in DPC micelles in D_2O plus 2 M ion concentration. The results show that the ^{35}Cl transverse magnetization in $CaCl_2$ solutions is composed of the two components (Fig. Fig. 66A). The ratio of the broad (or fast) component to the narrow (or slow) component is 1.66 (62% to 38%), calculated from the intercepts, exp(8.380)/exp(7.872). The fast transverse relaxation time (T_2') is 3.1 ms, and the slow transverse relaxation time (T_2'') is 8.6 ms. On the other hand, the ^{35}Cl transverse magnetization in NaCl solutions is a narrow and single-component decay since the broad component is only 6% of the narrow component as obtained from the ratio of intercepts, exp(6.259)/exp(9.045) (Fig. 6B). The transverse relaxation time (T_2) in NaCl is 14.1 ms, the same as the value measured from the line-width. Finally, the ^{35}Cl ion correlation time, τ_c, in $CaCl_2$ solution was calculated to be $2.3 \cdot 10^{-8}$ s. However, in DPC lipid micelles, two processes contribute to the correlation time, τ_c (unpublished data): one is the channel reorientation time, τ_r, which was obtained by ^{31}P-NMR to be $4.1 \cdot 10^{-8}$ s; the other is the ion occupancy time in the channel, τ_b. The off-rate constant from the doubly occupied state, k_{off}^w, for Cl^- ions in $CaCl_2$ is the reciprocal of τ_b, and the magnitude of k_{off}^w is determined from $(1/\tau_c - 1/\tau_r)$ to be $1.9 \cdot 10^7$/s. This result further confirms that the interaction between Cl^- ions and gramicidin A in DPC micelles does occur in $CaCl_2$, but not in NaCl; and it also shows that the k_{off}^w for Cl^- ion in $CaCl_2$ is close to that of Na^+ ion in gramicidin A in DPC micelles ($2.8 \cdot 10^7$/s) (Jing and Urry, unpublished data) and in LPC vesicles ($2.1 \cdot 10^7$/s) [25]. However, Cl^- ions cannot pass through the GA channel forming a measurable anion current because Cl^- ions can only enter and exit the channel as the $Ca^{2+} \cdots Cl^-$ ion pair.

3.7. Implication for the Interaction between $CaCl_2$ and the GA channel

By two independent NMR studies, it is shown clearly that the Cl^- ion binds in the channels. By the carbon-13 induced carbonyl carbon chemical shift, Cl^- binding is at the position of the carbonyl group of Trp^{11} residue about 5.5 Å away from the mouth of the channel and Ca^{2+} binding is at the position of the carbonyl group of Trp^{15} residue about 2.5 Å away from the entrance of the pore when the Cl^- ion and the Ca^{2+} ion enter the GA channel as an ion pair. Normally anions cannot pass through the GA channel to form a measurable single-channel current [9]; this was confirmed by the ^{13}C chemical shift studies in NaCl solution, in which no negative chemical shift induced by Cl^- ions was observed [8,14]. It was also supported by the ^{35}Cl line-width study by the NaCl titration. The reasons for the GA channel discriminating against anions were proposed as follows: in terms of the libration theory, the structure of the GA channel is energetically favorable to the interaction site for cations, but not for anions because the carbonyl oxygens with their large negative dipoles rotate into the channel; and the entrance to the channel itself presents a negative potential attracting cations and repulsing anions [12,28] again due to the carbonyl oxygens. For the same reason, the theoretical calculations proposed a high energy barrier at the channel entrance that blocks anion transport [29]. Interestingly, the interaction between Cl^- ions and gramicidin A in DPC micelles at the position of Trp^{11} residue is observed when a Cl^- ion is paired with a Ca^{2+} ion. This observation suggests that the unfavorable binding energy and the energy barrier at the channel entrance for anions could be modified by the Ca^{2+} ion coming with the Cl^- ion making it possible for an anion to enter the GA channel.

The evidence that Cl^- and Ca^{2+} ions as an ion pair bind and exchange rapidly with gramicidin A in DPC micelles is considered to be related to the phenomenon that $CaCl_2$ blocks the single-channel current for monovalent cations and disrupts the linear current–voltage relationship

[10]. The critical factors of blocking the action of the GA channel by adding $CaCl_2$ are suggested to be that: (1) according to the analysis of the ^{13}C chemical shifts, the binding constants, K_b^t and K_b^w, for gramicidin A in DPC micelles exhibited by monovalent cations are in the ranges 40–80/M and 2.5–4/M, respectively [30]; however, the constants for $CaCl_2$ are 120–180/M and 6/M, i.e., Ca^{2+} and Cl^- ions as an ion pair have a more favorable interaction with the GA helix than the monovalent cations do. (2) The center of the binding site of the GA channel for monovalent cations is between the carbonyl groups of the Trp^{11} and Trp^{13} residues and the distance between the two binding sites is about 16 Å [30]; and, a Cl^- ion is bound near the carbonyl group of the Trp^{11} residue and the distance between the two bound Cl^- ions is about 15 Å; hence, Cl^- ions when ion-paired with Ca^{2+} and monovalent cations have about the same binding position in the GA channel. Therefore, it is argued that Ca^{2+} and Cl^- ions as an ion pair have a stronger ability to compete with monovalent cations for the binding interaction and the probability of the monovalent cations to occupy the binding sites in the GA channels will be largely decreased when $CaCl_2$ is added to a monovalent ion solution. As a result, the single-channel current of monovalent cations is decreased, and the current–voltage curve is saturated.

Acknowledgements

This work was supported by grant No. N00014-89-J-1970 from the Department of the Navy, Office of Naval Research. The authors would like to express appreciation to T. Parker for the purification of the gramicidin A sample and to K.A. Prasad for having synthesized the carbon-13 labeled gramicidin A molecules.

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