^{23}Na-NUCLEAR MAGNETIC RESONANCE INVESTIGATION OF GRAMICIDIN-INDUCED ION TRANSPORT THROUGH MEMBRANES UNDER EQUILIBRIUM CONDITIONS

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ABSTRACT A technique for investigating the gramicidin-facilitated transport of Na^+ ions across lipid bilayers of large unilamellar vesicles under the condition of ionic equilibrium has been developed using a combination of heat incubation of the gramicidin with the vesicles and ^{23}Na-nuclear magnetic resonance (NMR) spectroscopy. Isolation of the two ^{23}Na-NMR signals from the intra- and extravesicular Na^+ with the shift reagent, dysprosium (III) tripolyphosphate, allows the equilibrium flux of Na^+ through the gramicidin channels to be detected and treated as a two-site exchange process. This study indicates that the transport of Na^+ through gramicidin channels is second order with respect to the gramicidin concentration.

INTRODUCTION

One of the most highly characterized channel-forming ionophores is the linear pentadecapeptide gramicidin. The tertiary structure of the active, membrane-bound channel has been established as being a $\beta^{6.3}$ helix consisting of two antiparallel monomers joined head to head by six intramolecular hydrogen bonds (Urry, 1971; Szabo and Urry, 1979; Bamberg et al., 1977; Weinstein et al., 1979, 1980). Once formed, the dimer's length of 26 Å is sufficient to span a lipid bilayer. The channel forms a 4-Å pore through the membrane. Lined with the carbonyl oxygens of the amino acid residues, the pore is permeable only to single file monovalent cations and water molecules (Myers and Haydon, 1972; Urban et al., 1980; Finkelstein and Andersen, 1981). Patch clamp measurements, sensitive enough to monitor single channel events, indicate that the gramicidin monomers dimerize for measurable lifetimes to form the active dimeric channels (Andersen, 1984). A gramicidin channel has the ability to transport 2.6×10^6 ions per second in the absence of an electrical potential difference across the membrane (Finkelstein and Andersen, 1981). This represents the maximum Na^+ ion conductance, assuming the Na^+ ion concentration is high enough so that the channels always contain one Na^+ ion. Fig. 1 shows the gramicidin A dimer channel in a lipid environment and the steps involved in transport: (*a*) ion diffusion to the channel entrance, (*b*) ion binding at the channel entrance, (*c*) transport through the channel, (*d*) dissociation of the cation–channel complex, and (*e*) diffusion away from the channel.

Through the use of a chemical shift reagent, one can observe the ^{23}Na-nuclear magnetic resonance (NMR) signals of the internal and external aqueous sodium ion pools present in cell suspensions (Gupta and Gupta, 1982; Balshi et al., 1982; Ogino et al., 1983; Boulanger et al., 1985) as well as in model vesicle systems (Pike et al., 1982; Riddell and Hayer, 1985; Hunt and Veiro, 1986). Once the two sodium signals are separated, changes in them may be used to study the kinetics of transmembrane ion transport. Artificial membranes in the form of vesicles are useful for studies concerning ion flux because of their inherent simplicity. Produced from synthetic or reconstituted lipids, vesicle membranes are highly ordered and well characterized in comparison to the complexity of biological cell membranes. This simplicity allows one to easily monitor small perturbations in the ^{23}Na-NMR spectrum produced by the addition of an Na^+ transporting agent, such as gramicidin, to the membrane separating the internal and external aqueous Na^+ pools.

Using a system of large unilamellar vesicles (LUV) in which the external sodium ions have been dialyzed away and replaced with lithium ions, Pike et al. (1982) have shown that the presence of small amounts (nM) of the channel-former gramicidin stimulates rapid efflux of sodium ions down their concentration gradient. Kinetic measurements of the two $^{23}Na^+$ NMR signals arising from the internal (Na_{in}) and external (Na_{out}) sodium ion pools of these systems show the area of the Na_{out} resonance signal to rapidly increase at the expense of the Na_{in} signal, in response to the gramicidin incorporated into the mem-

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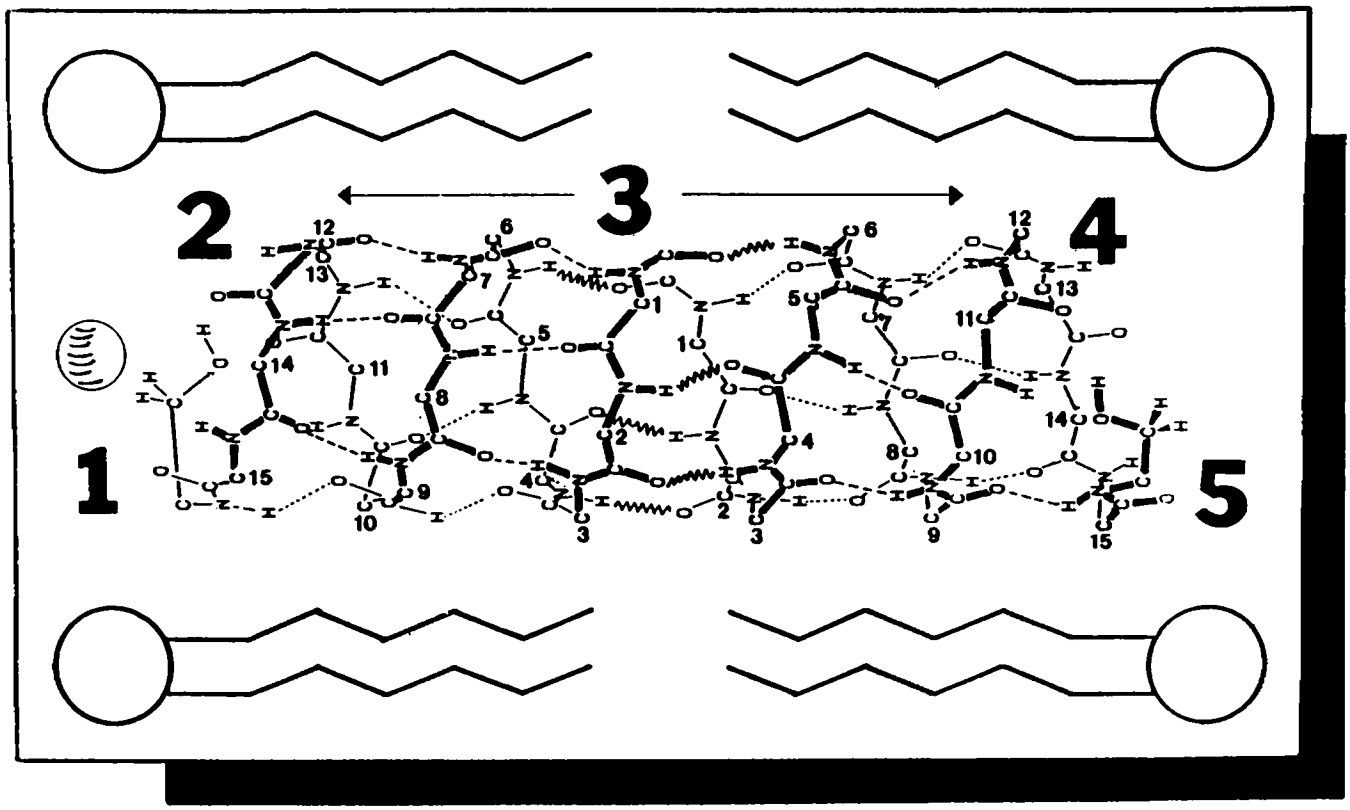

FIGURE 1 A schematic drawing of a gramicidin dimer within a lipid environment showing an approaching cation and the positions of the five steps involved in the transport process. The gramicidin dimer is that of Etchebest and Pullman (1985). (Permission to reproduce the gramicidin channel granted by Adenine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

brane. As depicted in Fig. 2, where the area of the Na_{in} signal is plotted as a function of time, time-course efflux measurements of this type show that the transmembrane channels formed by gramicidin are highly active transporters of Na^+. The graphical representation of the time-course change in peak areas stimulated by gramicidin shows the curve produced by the data to be biphasic (Pike et al., 1982). Unfortunately, the initial efflux rate of Na^+ through the gramicidin channels is too rapid to be accurately monitored with NMR techniques (curve *A*). However, as the efflux rate slows, the curve gently slopes toward ionic equilibrium (curve *B*). The slower efflux region of the time-course curve is probably the result of vesicle fusion and/or gramicidin exchange between vesicles. A discussion of this latter phenomenon will be presented in a subsequent paper.

With the ^{23}Na-NMR technique, Riddell and Hayer (1985) have recently demonstrated the monensin-mediated transport of sodium ions, under equilibrium conditions, through the walls of LUV to be dynamic on the NMR time scale. The line broadening in the $^{23}Na^+$ NMR spectrum observed with the incorporation of monensin into the LUV is consistent with a dynamic exchange process between Na_{in} and Na_{out}. The transport is first order in monensin and occurs by the formation of a 1:1 Na^+–ionophore complex.

We wish to report the preliminary results of the ^{23}Na-NMR spectroscopic investigation of the gramicidin-mediated transport of sodium ions across the walls of large unilamellar vesicles under conditions of ionic equilibrium. This technique is potentially very useful in probing a

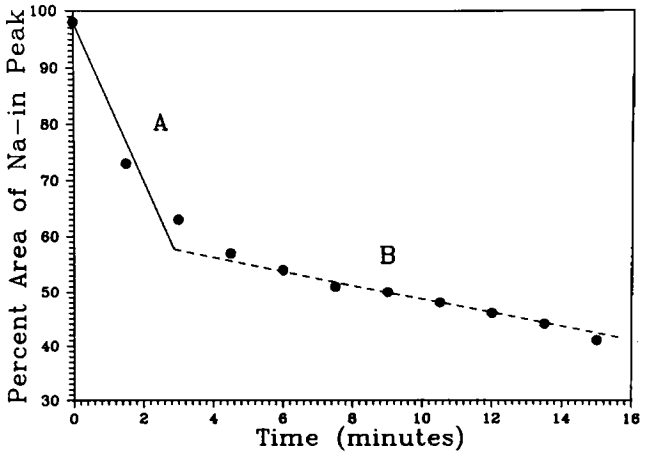

FIGURE 2 The time-course change in the relative area of the $^{23}Na_{in}$ peak of a LUV suspension after the injection of 5 μl of a gramicidin A-methanol solution. The LUV were prepared such that initially $[Na^+_{in}]$ = 100 mM; $[DyPPP_{in}]$ = 3 mM; $[Na^+_{out}$ = 4 mM; $[K^+_{out}]$ = 100 mM. The final analytical concentration of gramicidin was 100 nM.

number of the properties of the gramicidin channel. The equilibrium experiment allows one to study (*a*) the temperature dependence of the incorporation of functional gramicidin dimers into the lipid bilayers, (*b*) the relative equilibrium exchange rate of NMR active monovalent cations through the gramicidin channel, (*c*) bi-ionic flux ratios of different cations, (*d*) the effect of single amino acid substitution in gramicidin on the transport rate and the associated thermodynamic parameters, (*e*) effects, such as, UV-photodeactivation of gramicidin facilitated ion transport, and (*f*) lipid or lipid constituent effects on channel incorporation and transport.

MATERIALS AND METHODS

Materials

Gramicidin D samples (Sigma Chemical Co. [St. Louis, MO] and/or U.S. Biochemical Corp. [Cleveland, OH] 85% A, 15% B and C) were prepared in 2,2,2-trifluoroethanol (TFE) (Sigma Chemical Co.) and injected into the vesicle suspensions with a microliter syringe. Phosphatidylcholine (PC) and phosphatidylglycerol (PG) (Sigma Chemical Co.) were purchased as chloroform solutions and used without further purification. The saline solutions were prepared with doubly deionized water from reagent grade salts (Fisher Scientific Co., Pittsburgh, PA). The sodium salt of the anionic shift reagent dysprosium (III) tripolyphosphate (DyPPP) was synthesized using an in situ reaction (Gupta and Gupta, 1982). This shift reagent induces a high field shift to the NMR signals of the sodium ions with which it is in contact (Gupta and Gupta, 1982; Chu et al., 1984).

Vesicle Preparation

PC and PG were used to produce LUV by the reverse phase evaporation technique of Szoka and Papahadjopoulos (1978). Each milliliter of LUV was produced using a total of 66 μmol of lipids in a 4:1 PC/PG molar ratio. The solutions inside and outside of the vesicles contained 100 mM NaCl and were buffered with K_2HPO_4/H_3PO_4 to a pH of 8.2. Filtration of LUV through 0.4-μm polycarbonate membranes produces a reasonably homogeneous population of vesicles with an average diameter of 400–500 nm (Szoka et al., 1980). ^{23}Na-NMR (as described below) shows this type of vesicle preparation to routinely encapsulate 10–15% of the total aqueous volume inside of the LUV.

To incorporate gramicidin into the LUV, the desired quantity of a stock gramicidin/TFE solution (0.6–1.0 mM) was injected into 0.5-ml samples of the filtered vesicles. These samples were then agitated with a vortex mixer and immediately placed into a 50°C circulating water bath. After a 90-min incubation period, the LUV were removed from the bath and cooled to room temperature. At this point the samples were diluted 1:1 with an aqueous solution of DyPPP such that the final concentration of the shift reagent present on the outside of the vesicles was 5 mM. The solution was then allowed to equilibrate for 1 h before data acquisition.

NMR Measurements

The resonance shift experienced by sodium nuclei in the presence of DyPPP is a function of the Na^+/DyPPP ratio and has been previously characterized (Chu et al., 1984). The DyPPP present on the outside of the vesicles allows a spectral splitting to be observed for the signals arising from the internal and external sodium ions. Integration of these two signals gives a direct measurement of the total amount of sodium ions encapsulated by the vesicle membranes and thus defines the population of sodium ions residing in each of the two magnetic environments. ^{23}Na spectra were recorded at 23.65 MHz on a JEOL FX90Q spectrometer using a 10-mm multinuclear probe. Typically, 1000 FIDS were accumulated per data set at a probe temperature of 25°C.

The rotating frame selective inversion-recovery experiments were performed at 9.4T on an AM400 WB spectometer (Bruker Instruments, Inc., Billerica, MA). Each spectrum consists of 60 accumulations of 2K points, covering a sweep width of 5,000 Hz, with a signal acquisition of 0.2048 s, a 90° pulse width of 18 μs, and a t_1 value of 350 μs. A total of 18 mixing times (50 μs to 300 ms) was used. For the two-dimensional NOESY experiment, 64, 512-word spectra were collected using a mixing time of 10 ms. The shortest evolution time (t_1) was 3 μs. The t_1 dimension was zero-filled to obtain a 512 × 512 data matrix, which was then two-dimensionally Fourier transformed in the power mode to give the displayed spectra.

UV Irradiation

To monitor the effect of photolysis on cation transport through the channel, gramicidin was first incorporated into a 1-ml sample of LUV with heat incubation. After obtaining the initial ^{23}Na-NMR spectrum, the sample was transferred to a 1 ml, narrow, quartz cuvette. This cuvette was positioned in the beam of an unfiltered 150-W xenon lamp (Schoeffel Instrument Co., Westwood, NJ) such that the whole sample was irradiated. After the indicated irradiation time, the sample was returned to the NMR tube and the ^{23}Na-NMR spectrum was recorded. The process was repeated a number of times.

RESULTS

A necessary condition for the time-course measurements described by Pike et al. (1982) is the replacement of the external Na^+ of the vesicle suspensions with a suitable counter ion such as K^+ or Li^+ so that a concentration gradient exists across the membranes. When such a chemical potential is present, nanomolar amounts of gramicidin are seen to readily incorporate into the membrane and stimulate the rapid efflux of Na^+ across the lipid bilayers as shown in Fig. 2. To initiate transport in these systems, all that is required is the injection of a microliter aliquot of gramicidin into the dialyzed vesicle suspension. ^{23}Na-NMR spectra show immediate changes in the signal intensities, indicating that gramicidin readily incorporates into the lipid membranes forming functional channels. This is not the case when a gramicidin solution is injected into a sample of LUV that is at ionic equilibrium.

Initial attemps to observe line-broadening under equilib-

TABLE I
EFFECT OF INCORPORATION TIME ON THE WIDTH OF THE $^{23}Na_{in}$ SIGNAL

[Gramicidin]*	Half-height line-width of Na_{in} signal (Hz)‡			
	Time = 0	30 min	60 min	90 min
μM				
0	17	17	17	17
2.45	—	19	24	25
3.68	—	21	34	33
4.90	—	29	43	43
7.96	—	47	79	81
9.80	24	56	111	112

*Final analytical monomer concentration of gramicidin D in 1-ml LUV samples.
‡23.65-MHz spectra taken at a probe temperature of 25°C after incubation of the samples at 50°C for the indicated times.

rium conditions caused by the injection of gramicidin into the vesicle solution showed no effect upon the two sodium NMR signals. However, experience with micellar-packaged gramicidin systems (Urry et al., 1979; Hinton et al., 1986; Shungu et al., 1986) where the incorporation of active channels requires incubation of the gramicidin–lipid mixtures at elevated temperatures, suggested a similar incubation might be necessary with the LUV system under conditions of ionic equilibrium. The data presented in Table I show that the injection of a TFE/gramicidin solution, without incubation, has little effect upon the line-width of the Na_{in} signal. However, it can be seen that when the LUV samples are heated at 50°C, the line-width of the internal Na^+ signal increases after 30- and 60-min incubation time. Little change is seen after further heating. These preliminary measurements show that the incorporation of functional transmembrane gramicidin channels into the walls of LUV under conditions of ionic equilibrium is not spontaneous but requires an elevated temperature incubation period. It appears that the barrier to the incorporation of gramicidin into a lipid environment is overcome by transmembrane potential effects in the non-equilibrium experiment and by thermal effects under ionic equilibrium conditions.

Once incorporated into the bilayers of LUV, gramicidin induces line-broadening of both signals in the ^{23}Na-NMR spectrum (Fig. 3). The site of lower population (Na_{in}) can be seen to broaden more rapidly in response to increasing amounts of gramicidin. Half-height linewidth measurements of this signal allow the mean lifetime of the Na^+ inside of the LUV (τ_{in}) to be determined for each gramicidin concentration through the relationship $1/\tau_{in} = \pi\upsilon_{in}$ (Riddell and Hayer, 1985; Sandstrom, 1982). The extent of this broadening appears to be consistent with a dynamic exchange process between Na_{in} and Na_{out} through the transmembrane channels formed by gramicidin. It was found that $1/\tau_{in}$ is a nonlinear function of gramicidin concentrations, as shown in Fig. 4.

The nonlinearity of the data represented in Fig. 4 suggests that the transport of sodium through the walls of LUV is not first order with respect to the gramicidin concentration present in the membranes. In the present system, where the gramicidin concentration is varied and the sodium ion concentration is constant, $1/\tau_{in}$ can be described by the simple kinetic expression: $1/\tau_{in} = k$ [Gram]^n, where n is the kinetic order of the transport reaction with respect to the gramicidin monomer concentration ([Gram]) and k is the apparent n_{th} order rate constant for the process.

The kinetic order of this reaction may be obtained from the slope of a plot of $-\log \tau_{in}$ as a function of log [Gram]. Plots for the data obtained with gramicidin D in these LUV systems are linear over the range of gramicidin concentrations studied and produce apparent kinetic orders of 1.7–2.2 (Fig. 5). A kinetic order of 2 is consistent with the fact that two gramicidin monomers are required to dimerize and form a channel before sodium ion transport can take place.

If the transport is indeed second order with respect to the gramicidin concentration, then $1/\tau_{in}$ should be a linear function of the square of the gramicidin monomer concentration. In Fig. 6, $1/\tau_{in}$ as a function of $[Gr]^2$ produces a straight line having a correlation coefficient of 0.998.

The spectra of Fig. 3 suggest that the dynamic NMR technique is applicable to the measurement of gramicidin

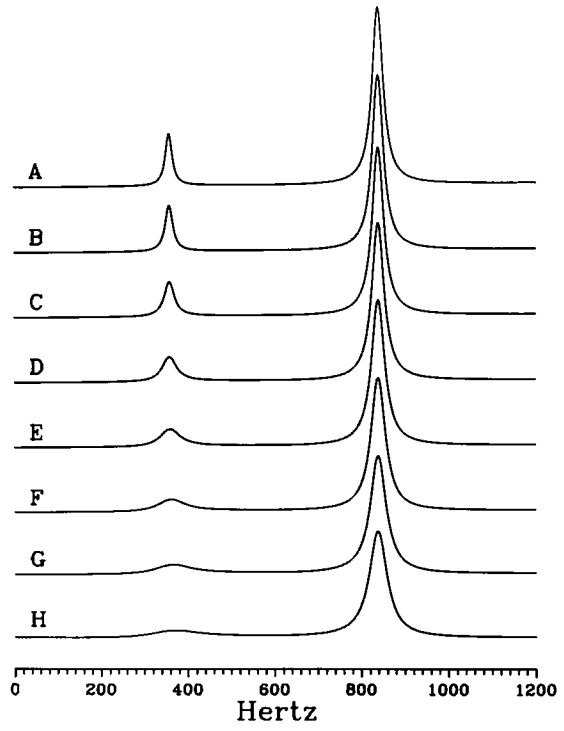

FIGURE 3 Experimental ^{23}Na-NMR spectra of equilibrium LUV incorporated with (*A*) 0, (*B*) 1.4, (*C*) 2.8, (*D*) 3.2, (*E*) 4.6, (*F*) 6.0, (*G*) 7.4, and (*H*) 9.0 μM gramicidin D.

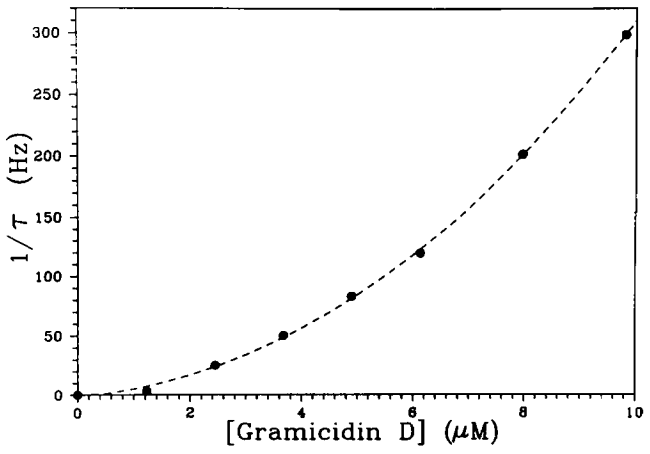

FIGURE 4 The mean lifetime ($1/\tau_{in}$) of the $^{23}Na_{in}$ spin site as a function of gramidcidin D concentration. The dashed line is included only to guide the eye.

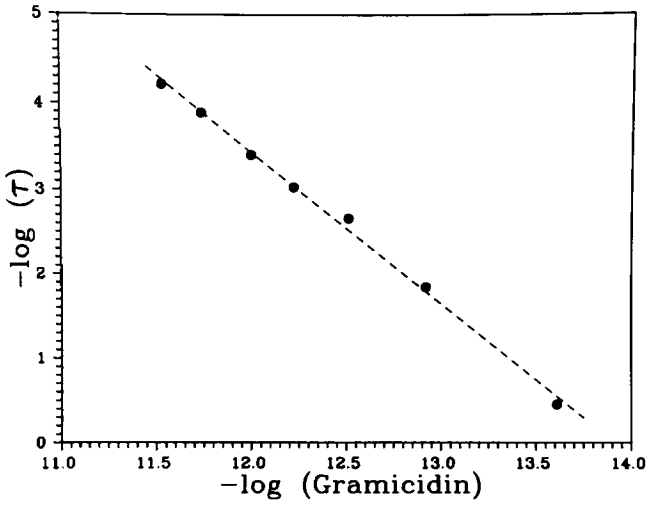

FIGURE 5 Log of the mean lifetime of the Na^+ inside of the LUV ($1/\tau_{in}$) versus log of the gramicidin concentration. Linear regression of the data results in the dashed line which has a slope of 1.8 with a correlation coefficient of 0.998.

facilitated flux of sodium ions under equilibrium conditions. The obvious advantage of this technique over the kinetic method is that there is no significant time limit in obtaining the data. The lack of any time constraint in data collection means that the exchange rate of any cation, such as K^+, that is transported through the gramicidin channel may be directly determined even though the inherent sensitivity of the nucleus of interest to the NMR experiment is very poor.

One obvious question that might be asked about the line-broadening observed in these experiments is that regarding the integrity of the vesicles. If the LUV membranes were to rupture or to become "leaky" during the incubation period, the Na_{in} signal intensity would be expected to diminish and the peak broaden and shift to higher field due to the infiltration of the shift reagent. A number of different techniques were used to show that the perturbations of the sodium signals arise solely from ion transport rather than from an artifact due to vesicle instability.

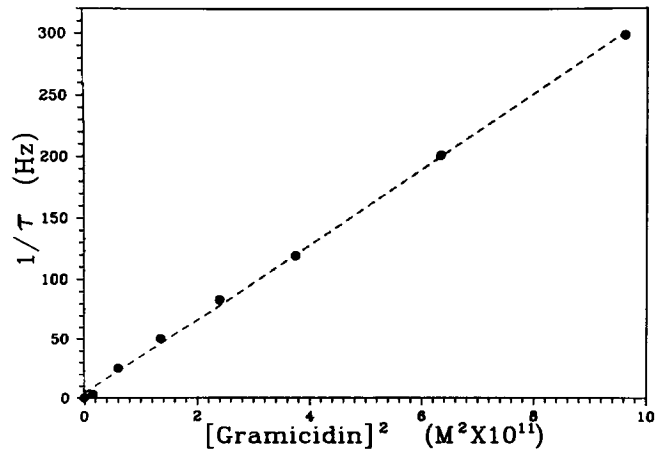

FIGURE 6 The mean lifetime of the Na^+ inside of the LUV ($1/\tau_{in}$) as a function of the squared gramicidin concentration.

(A) Thermal Effect

Basic rate theory, as well as dynamic NMR theory, predicts that the rate of an exchange reaction should be a function of the temperature. In the present case, where one is measuring the equilibrium exchange between Na_{in} and Na_{out}, transport through the gramicidin channels should be a function of temperature. As transport through the channel increases, the mean lifetime (τ) of the sodium ions decreases causing the spectral lines to broaden and shift towards coalescence. In Fig. 7 the effect of elevated temperature of the spectrum of a gramicidin-incorporated LUV suspension is illustrated. The two peaks obtained at a temperature of 25°C are seen to approach coalescence at a temperature of 60°C. Cooling the sample back to 25°C results in the production of a spectrum identical with the initial one. The increase in the line-widths is consistent with an increase in the exchange rate process. It should be noted that the signal for Na_{out} at 60°C shifts due to the

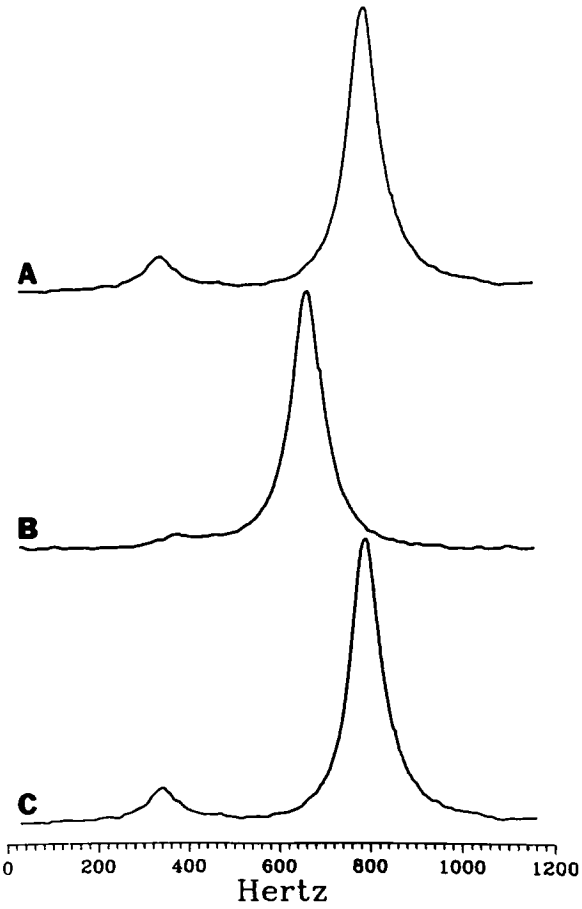

FIGURE 7 Effect of elevated temperature on the ^{23}Na signals of LUV containing 6.7 μM gramicidin D. Top spectrum (*A*) taken at a probe temperature of 25°C, the middle (*B*) at 60°C, and the lower (*C*) at 25°C.

exchange process and to a decrease in the interaction with the shift reagent. That the signals return to their initial intensities and relative chemical shifts indicates that the vesicles retain their integrity at elevated temperatures. Line shape analysis of the spectra as a function of temperature also provides one the opportunity of determining the thermodynamic parameters for the exchange process.

(B) Photolysis Effect

Recently, the decay of gramicidin fluorescence, resulting from ultraviolet exposure, has been correlated with the decrease in channel conductance in gramicidin-containing planar bilayer membranes under the same conditions of illumination (Jones et al., 1986). It was suggested that the loss of membrane conductance arises from the inactivation of the gramicidin channels due to photochemical modifications of the channel tryptophans. These results indicate that UV-photolysis might be a means of deactivating the incorporated gramicidin channels in the LUV systems and, hence, slowing the rate of exchange of Na^+. The photolytic deactivation of functional gramicidin channels in vesicles does diminish the transport rate as can be seen in Fig. 8.

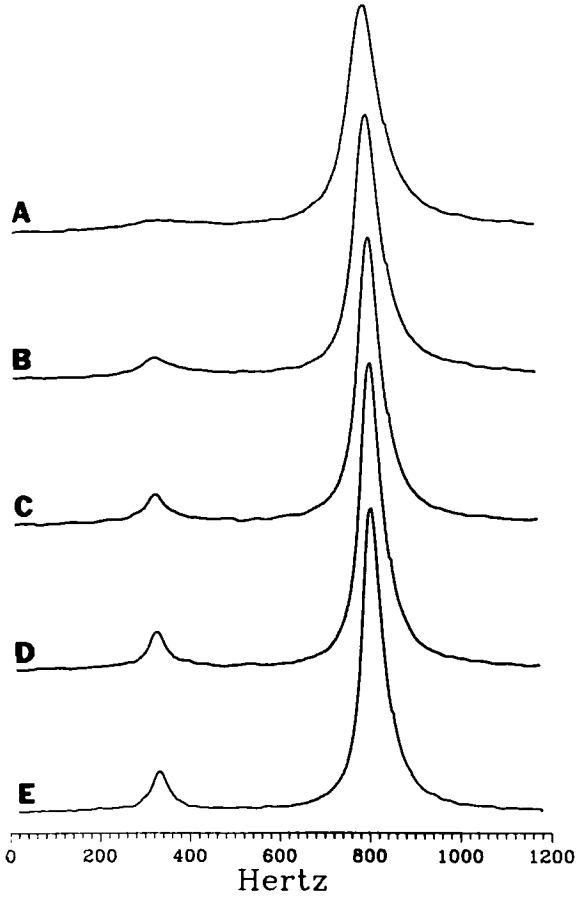

FIGURE 8 Effect of UV-photolysis on the ^{23}Na signals of LUV containing 19.2 μM gramicidin D. Spectrum (*A*) taken before UV-irradiation and spectra (*B–E*) after 2, 4, 6, and 8 min, respectively, in the beam of a 150-W xenon lamp.

This channel deactivation is seen to reverse the line-broadening caused by the incorporated channels. Spectra *A–E* in Fig. 8 show line-narrowing as a function of UV irradiation time. Again, changes in line-widths observed in the equilibrium experiment appear to be caused by Na^+ exchange rather than vesicle degradation. This experiment also shows that the transport properties of the gramicidin channel incorporated into a lipid environment can be modified in ways other than through temperature changes or synthetic modification of gramicidin.

(C) Magnetization Transfer NMR

There are two NMR techniques, inversion-transfer and two-dimensional exchange, that also provide a way to directly detect the exchange between Na_{in} and Na_{out} in a vesicle system that contains gramicidin channels. The inversion-transfer experiment is a modification of the more familiar saturation transfer experiment (Forsen and Hoffman, 1963). If the transport rate constant through the gramicidin channel is at least comparable to the relaxation rate of the $^{23}Na^+$ ion, magnetization of one spin state, $^{23}Na_{out}$, can be transferred to the other spin state, $^{23}Na_{in}$ (Forsen and Hoffman, 1963). In the inversion-transfer experiment, the magnetization of the $^{23}Na_{out}$ state is inverted 180 degrees followed by a variable delay time before the spectrum is recorded. This pulse sequence causes the NMR signal of the $^{23}Na_{in}$ state to change with respect to the delay time. As can be seen in the spectra of Fig. 9 *A*, the magnetization of the $^{23}Na_{in}$ state goes through

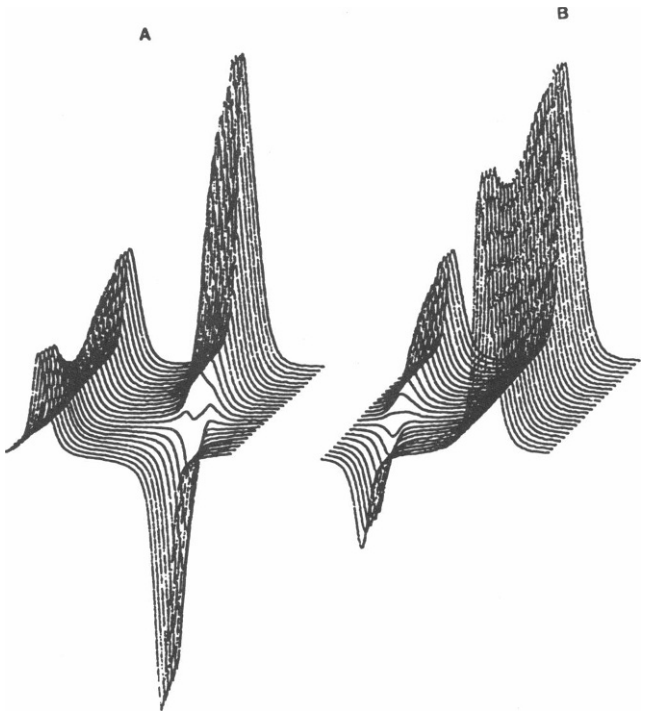

FIGURE 9 Inversion-transfer experiment. (*A*) Outside signal inverted. (*B*) Inside signal inverted.

a minimum for the delay time at which the magnetization of the $^{23}Na_{out}$ signal is at a minimum in the plane perpendicular to the direction of the applied magnetic field. The same experiment can be performed in a reverse manner (i.e., inverting the magnetization of the $^{23}Na_{in}$ state and after the magnetization decrease of the $^{23}Na_{out}$ state, as shown in Fig. 9 *B*). It is obvious from these experiments that magnetization is transferred from one state to the other by fast cation transport through a gramicidin channel.

An even more elegant NMR technique for the detection of exchange of Na^+ between Na_{in} and Na_{out} through the gramicidin channel is the two-dimensional exchange experiment (Wüthrich, 1986). The essence of this experiment involves the observation of cross-peaks if exchange occurs and no cross-peaks in the two-dimensional spectra in the absence of exchange. Fig. 10 *A* shows the results of the two-dimensional exchange experiment on the vesicle system that does not contain gramicidin incorporated into the membrane. The absence of cross-peaks indicates no exchange between $^{23}Na_{in}$ and $^{23}Na_{out}$. However, the same experiment with the vesicle system that has gramicidin incorporated into the membrane shows cross-peaks (see Fig. 10 *B*), which provides unequivocal evidence for the presence of exchange between the two magnetic environments. It is important to note that not only do the inversion-transfer (Alger and Shulman, 1984) and the two-dimensional exchange (Meier and Ernst, 1979) experiments provide evidence in favor of the transport process; they may be also expanded to determine the transport rate of the cation through the gramicidin channel.

CONCLUSIONS

To construct a suitable kinetic model for ion transport through the gramicidin channel as depicted in Fig. 1, one must be able to independently characterize the individual steps involved in the process. Recently, T1-205 NMR spectroscopy has proven to be very useful for investigating step 2 shown in Fig. 1. Equilibrium binding constants and the thermodynamic parameters associated with cation binding have been obtained for monovalent and divalent cations (Hinton et al., 1986; Shungu et al., 1986). Having devised techniques for obtaining quantitative information about the binding process, an experimental approach was sought for investigating the transport rate through the channel (i.e., step 3).

The elegant time-course measurements of Pike et al. (1982) showed that ^{23}Na-NMR could be used to monitor the transport activity of gramicidin. Unfortunately, the rapidly changing ^{23}Na-NMR signal intensities encountered in dialyzed LUV suspensions place rather severe limitations on the ability to obtain initial transport rates. However, for a system at ionic equilibrium, the resulting ^{23}Na-NMR signals are static. Therefore, no time constraint is present to limit the amount of data that can be accumulated.

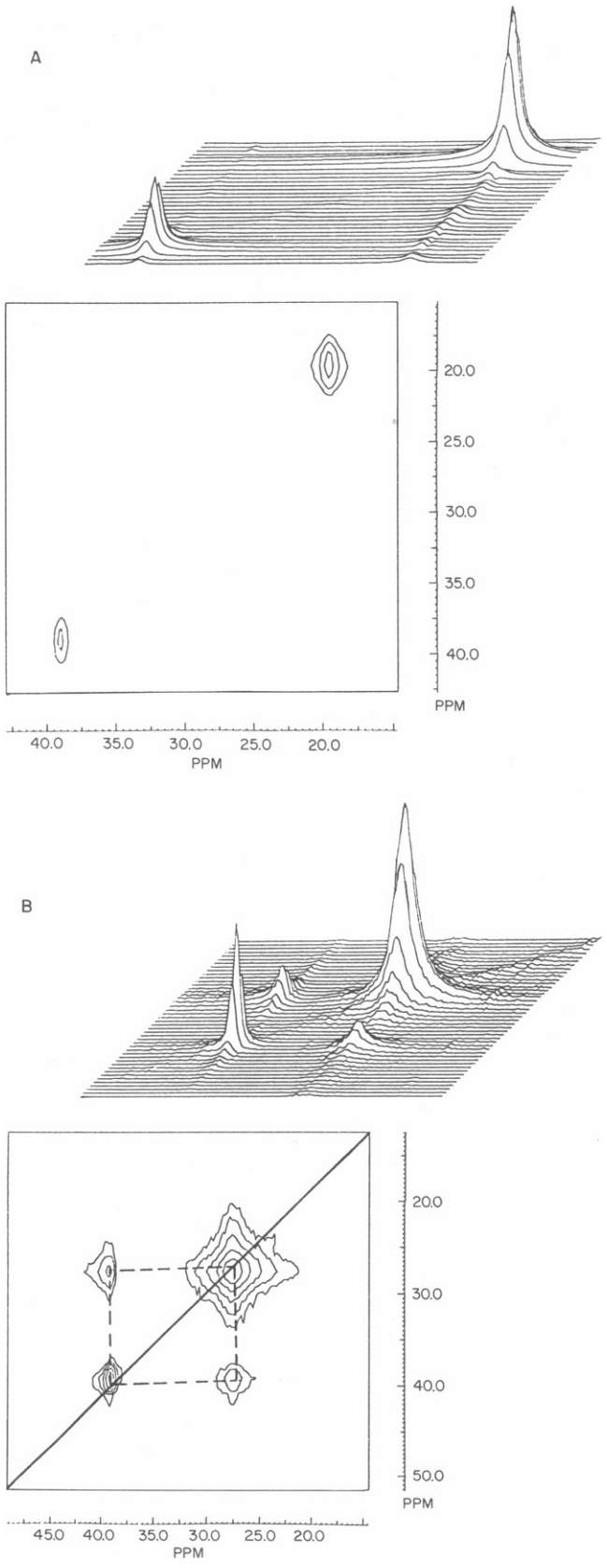

FIGURE 10 Two-dimensional exchange experiment. (*A*) No gramicidin; (*B*) gramicidin incorporated into the membrane.

Current studies in this lab indicate this technique to be applicable to the measurement of relative transport rates of differing cations in LUV systems containing two or more NMR active cations. Results to be presented in detail in the near future show the Na^+ ion traverses the gramicidin channel a factor of three to four times more quickly than does Li^+.

The results of this initial study with an ionic equilibrium LUV system show that: (*a*) the Na^+ movement through the gramicidin channel can be monitored only if the channels are incorporated into the lipid bilayer through heat incubation, (*b*) LUV are suitable for these studies since they are very stable during the time frame of the experiment, (*c*) the changes in the ^{23}Na-NMR signals with gramicidin arise from ion transport through the channel, and (*d*) the ^{23}Na-NMR technique can be used to monitor the effect of an externally applied optical stimulus to the photoactive residues of the gramicidin molecule.

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