BRIEF COMMUNICATION

Na^+ INTERACTING WITH GRAMICIDIN D

A NUCLEAR MAGNETIC RESONANCE STUDY

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ABSTRACT ^{23}Na nuclei in a milk-white emulsion composed of nonionic surfactant and higher alcohol in saline were characterized by single values of T_1 and T_2 and a single Larmor frequency. In the presence of small amounts of gramicidin D (Dubos), the relaxations of ^{23}Na were greatly accelerated, and the transverse relaxation was a sum of two decaying exponentials. But only a single T_1 was observed; it was roughly equal to the slow T_2. The slow T_2 accounted for about 40% of the total resonance intensity. The relaxation rates increased linearly with the increase of the gramicidin concentration. The absorption signal consisted of a narrow and a broad line, both centered at the same frequency. The present results suggest that nuclear magnetic resonance spectroscopy is a useful tool for studying the nature of ion-permeable channels of biological membranes, even when the channel has no ionizable groups.

INTRODUCTION

It is widely accepted that the nonionic pentadecapeptide gramicidin A forms cation-permeable channels when incorporated into subcellular membranes or artificial lipid membranes. The channel produced by gramicidin A exhibits very high transport rates for monovalent cations (1), comparable to those of the Na^+ channel of nerve fibers (10^7 sodium ions/s) (2). The gramicidin channel has received increasing attention as a model for permeation in ion channels postulated in various biological membranes (see, e.g., reference 3).

In this work, the interaction between sodium ions and gramicidin D (a mixture of gramicidins A, B, and C) was studied by ^{23}Na nuclear magnetic resonance (NMR)[1] spectroscopy. Gramicidin D was incorporated into nonionic emulsion particles dispersed in a large amount of NaCl solution. In the absence of gramicidin D, the ^{23}Na nuclei in this milk-white emulsion were characterized by single values of T_1 and T_2. When small amounts of gramicidin

[1] *Symbols and abbreviations used in this paper:* NMR, nuclear magnetic resonance; T_1, longitudinal relaxation time; T_2, transverse relaxation time; τ_c, correlation time for the fluctuation of electric-field gradients; ω_L, Larmor angular frequency.

D were added to the emulsion, the relaxations of ^{23}Na were greatly accelerated, and the transverse relaxation was no longer a simple exponential decay. The main features of the ^{23}Na resonance of this system were the same as those of biological tissue.

MATERIALS AND METHODS

Gramicidin D (Dubos) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Commercially available gramicidin D is a mixture of 70–85% gramicidin A, 5–10% gramicidin B, 7–20% gramicidin C, and gramicidin D (less than 1%) (4). The nonionic surfactants Emulgen 408 and Emulgen 430 (polyoxyethylene oleyl ethers; hydrophile-lipophile balance = 10.0 and 16.2, respectively) are kind gifts of Kao-Atlas Co., Ltd. (Tokyo, Japan).

Gramicidin D was dispersed in a small amount of ethyl alcohol and dissolved in a mixture of Emulgen 408, Emulgen 430, and oleyl alcohol (2%, 4%, and 2.4% by weight, respectively, as the final concentrations.) To this transparent solution, water and NaCl solution were added with agitation by a magnetic stirrer. The final concentration of NaCl was 140 mmol/kg sample. The stirring was continued for several hours, and finally a fairly stable, milk-white emulsion was obtained. The emulsion containing no gramicidin will be referred to as "basic emulsion."

The nuclear magnetic relaxation times of ^{23}Na were determined at 25 ± 0.1°C with a pulse NMR spectrometer (JEOL, Ltd., Tokyo, Japan; model JNM-FSE60A) operating at 15.8 MHz. Sample tubes with a spherical cavity were used. T_1 was determined from a 180°–τ–90° pulse sequence; T_2, from a 90°–τ–180° pulse sequence. The 90° pulse was 12–13 μs in duration. 128 or 256 transients were time-averaged. The acquisition time was 2 s. The absorption signal of ^{23}Na was obtained at 27 ± 1°C with a Fourier transform NMR spectrometer (JEOL, Ltd.; model JNM-FX100) operating at 26.4 MHz. The pulse width was 42 μs (90° pulse). The acquisition time was 1 s.

RESULTS AND DISCUSSION

In the basic emulsion, the transverse and longitudinal relaxations of ^{23}Na were simple exponential decays, and the absorption signal consisted of a single Lorentzian line (Fig. 1 and Table I). T_1 was approximately equal to T_2; they were about 30% shorter than those for dilute NaCl solutions.

When a small amount of gramicidin D was added to the basic emulsion, the relaxations of ^{23}Na were greatly accelerated, and the transverse relaxation was no longer an exponential decay (Fig. 1*a*). But we always observed only a single value of T_1 (Fig. 1*b*). The slow T_2 was roughly equal to, or slightly shorter than, T_1 observed in the same sample (Table I). The slow and fast T_2 accounted for about 40% and 60%, respectively, of the total resonance intensity, irrespective of the concentration of gramicidin D. The relaxation rates of ^{23}Na increased with the increase of the gramicidin concentration. The absorption signal consisted of a narrow and a broad line (Fig. 2). No shift was detected between the two component lines. These features of ^{23}Na resonance of emulsions containing gramicidin D are quite the same as those of biological tissue (5–9). In the presence of 0.8% gramicidin D, T_1 and T_2 values were comparable to those reported for fresh biological tissue (T_1 = 10–18 ms, slow T_2 = 9–14 ms, and fast T_2 = 0.7–1.1 ms [5–7]).

The ^{23}Na nuclei in those emulsions containing gramicidin D may be regarded as consisting of two populations, one of which (^{23}Na in "state A") is present in the bulk aqueous phase and characterized by fast tumbling ($\tau_c \ll \omega_L^{-1}$) and a single Larmor frequency; the other (^{23}Na in "state B") is the ^{23}Na nuclei interacting with gramicidin molecules. (Strictly speaking, we have a third state of ^{23}Na nuclei, which represents the ^{23}Na nuclei present

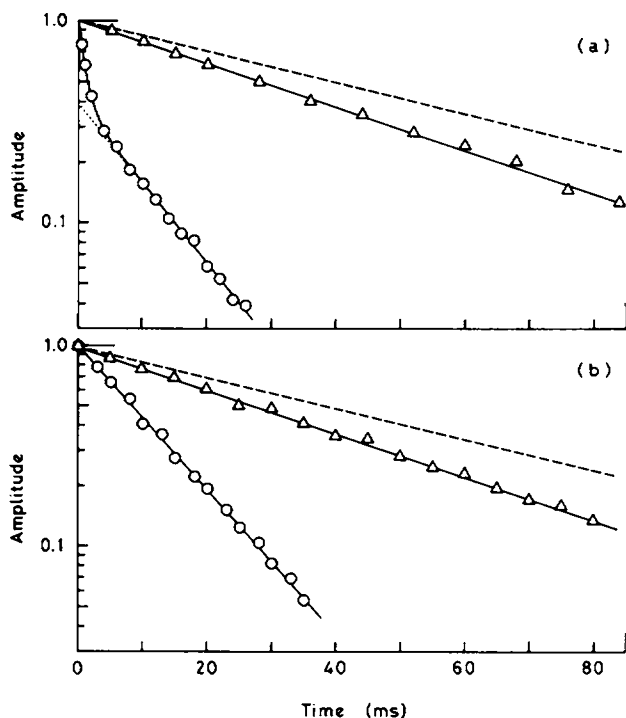

FIGURE 1 Transverse (*a*) and longitudinal (*b*) relaxations of ^{23}Na in an emulsion with or without gramicidin D. The composition of the emulsion is indicated in the Methods section. In *a*, the ordinate represents echo amplitudes per one ^{23}Na nucleus expressed in an arbitrary unit. In *b*, the initial amplitudes of the decay curves are independently normalized to unity; the ordinate represents $(A_\infty - A_\tau)$, where A_τ is the initial amplitude of the free induction decay after the 90° pulse at time τ. △, no gramicidin; o, 0.8% gramicidin. Broken lines are the decay curves for NaCl solution (140 mmol/kg solution).

TABLE I

^{23}NA RESONANCE OF AN EMULSION CONTAINING GRAMICIDIN D

Sample	T_1	T_2	Slow fraction	Absorption line
	ms	*ms*	%*	
NaCl solution, 140 mmol/kg solution	57.0 ± 0.9	56.7 ± 0.8	—	Single line
Basic emulsion	40.3 ± 1.0	40.8 ± 1.0	—	Single line
Basic emulsion + 0.4% gramicidin	18.3 ± 0.5	(slow) 16.7 ± 0.6 (fast) 2.1‡	39.0 ± 1.3	Broad and narrow lines
Basic emulsion + 0.8% gramicidin	12.2 ± 0.4	(slow) 10.9 ± 0.5 (fast) 1.1‡	39.3 ± 1.5	Broad and narrow lines

Data are values estimated by the least-squares curve fitting ± estimates of the standard error (degrees of freedom = 20–33; typically three samples).

* Percentage of the total resonance intensity.

‡ Values very inaccurate.

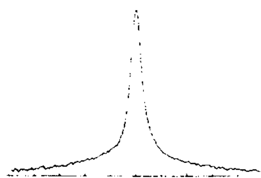

FIGURE 2 Fourier-transformed signal of ^{23}Na in an emulsion containing 0.8% gramicidin D. The composition of the emulsion is indicated in the Methods section. The solid line represents the Fourier-transformed signal, 2,300 transients being collected. The dotted lines are the two Lorentzian components of a calculated signal of the type: $g(\omega) = c'T_2'/[1 + T_2'^2(\omega - \omega_L)^2] + c''T_2''/[1 + T_2''^2(\omega - \omega_L)^2]$. The values of the parameters used are such that the equation $A(t) = c'\exp(-t/T_2') + c''\exp(-t/T_2'')$ shows the best least-squares fit to the envelope of the free induction decay signal in the time domain ($c' = 0.405$, $c'' = 0.595$, $T_2' = 13.2$ ms, $T_2'' = 1.2$ ms). The peak heights of the experimental and the calculated line are normalized to the same dimension. Scale markers on the abscissa are at intervals of 50 Hz. Under the same instrumental conditions, the apparent T_2 (T_2^*) of ^{23}Na in dilute NaCl solutions was 2–3% shorter than the true T_2 value.

near the surface of the emulsion particles, but not interacting with gramicidin D. The somewhat shortened relaxation times observed in the basic emulsion can be explained by the rapid exchange of ^{23}Na between the bulk phase and this third state. For simplicity, state A will be referred to as involving the third state.) The present results indicate that the exchange of ^{23}Na between states A and B is sufficiently rapid, and that the slow and the fast T_2 of ^{23}Na correspond to different energy transitions ($1/2 \leftrightarrow -1/2$ and $\pm 3/2 \leftrightarrow \pm 1/2$, respectively) of all the ^{23}Na nuclei. ^{23}Na in state B must be assumed to interact with electric-field gradients in such a manner that two different values of T_2 occur for the whole ^{23}Na population. The conditions to be fulfilled in this respect are (see reference 10): $\tau_c \gtrsim \omega_L^{-1}$ for ^{23}Na in state B, and/or an ordering of the electric-field gradients is present for ^{23}Na in state B. Ordering, as used here, means that that fraction of the ^{23}Na nuclei possesses, in the absence of exchange, more than one (or distributed) Larmor frequencies in the first-order effect of quadrupole inneractions.

Under the condition of rapid exchange, T_1 of ^{23}Na in the samples is given by

$$1/T_1 = p_A/T_{1A} + p_B/T_{1B}, \tag{1}$$

where T_{1A} and T_{1B} are the respective T_1 for states A and B in the absence of exchange, and p_A and p_B are the fractional populations of ^{23}Na in states A and B. The amount of gramicidin D in the present samples is very small as compared with that of Na^+, so that p_B and hence $(1/T_1 - 1/T_{1A})$ will be proportional to the concentration of gramicidin D. The result shown in Fig. 3 is in accord with this expectation.

According to the four-site model for the gramicidin A channel (3), one gramicidin channel can be occupied simultaneously by four cations. One gramicidin A channel is accepted as consisting of two molecules of gramicidin A (see, e.g., references 11–13; for the similarity among gramicidins A, B, and C, consult reference 13). Consequently, an upper bound of the amount of ^{23}Na in state B will be 9 mmol/kg sample for the emulsion containing 0.8% gramicidin D; when the affinity of Na^+ for the channel is taken into consideration, the upper bound will decrease to 4–5 mmol/kg sample, which corresponds to about 3% of the whole ^{23}Na population. It is noteworthy that such a small amount of ^{23}Na interacting with non-

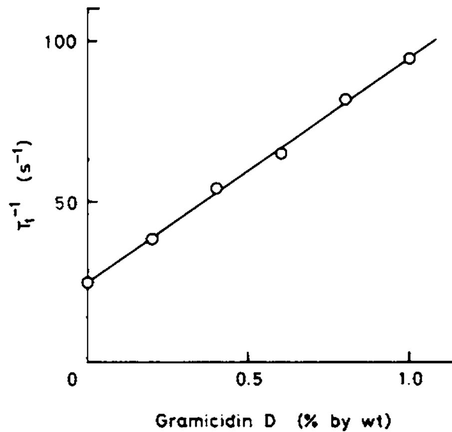

FIGURE 3 Effect of the gramicidin D concentration on T_1 of ^{23}Na.

ionic channel-forming substance is responsible for the fast relaxation rates and two distinct T_2 values comparable to those of ^{23}Na in biological tissue. The present results parallel a conclusion of a previous report (14) that the fraction of bound ^{23}Na in muscle tissue will be some few percent, at most, of the total ^{23}Na population.

The occurrence of two T_2 of ^{23}Na in biological materials has often been attributed to the presence of charged sites on particulate matter or larger macromolecules. The present results suggest that nonionic channels may also be responsible for the occurrence of two T_2 of tissue ^{23}Na, if such channels exist in subcellular membranes. It thus appears that NMR spectroscopy is a useful tool for studying the nature of ion-permeable channels postulated in various biological membranes, even when the channel has no ionizable groups.

Received for publication 24 July 1978 and in revised form 5 November 1978.

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