BBA 76004

PHOSPHORUS AND PROTON NUCLEAR MAGNETIC RESONANCE STUDIES IN SARCOPLASMIC RETICULUM MEMBRANES AND LIPIDS

A COMPARISON OF PHOSPHATE AND PROTON GROUP MOBILITIES IN MEMBRANES AND LIPID BILAYERS

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(Received March 6th, 1972)

SUMMARY

Phosphorus nuclear magnetic resonance spectra, recorded at 101 MHz, of rabbit sarcoplasmic reticulum membrane fragments and lipid extracts of these membranes are characterized by single broad lines (approx. 1 kHz half width). The line width may be, in part, field dependent. The estimated residual (zero field) width is interpreted in terms of local proton dipolar fields, randomly modulated in time. The correlation time for such motion is estimated to be on the order of 10^{-5} s. This motional time constant for the phosphate head group in membranes and lipid dispersions is approx. $10^2 - 10^3$ slower than that characteristic of portions of the hydrocarbon chains and choline methyl groups.

The phosphorus spectra, in contrast to the proton NMR spectra, is insensitive to chemical alteration of the membrane protein. Likewise, in lipid extracts, the accompanying increased fluidity in some proton regions of the lipid molecules is not manifest in the phosphate group. While increasing the temperature to 40 °C narrows the ^{31}P line width there is no evidence of a highly mobile state for the phosphate group like that found for the choline group.

INTRODUCTION

In previous work, proton nuclear magnetic resonance (NMR) spectra of sarcoplasmic reticulum vesicles indicated that in this membrane the molecular motion of the majority of lipid hydrocarbon chains is characterized by a correlation time, τ_c^H, of 10^{-8} s. These values, were calculated approximately by comparing the observed NMR line width with calculated and observed values for rigid lattice limit of the proton NMR line second moment[1]. Considering that the time constant for Brownian motion of sarcoplasmic reticulum vesicles of diameter 0.1–0.2μm

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is approx. 10^{-3} s, the observed NMR line width was attributed to thermal motion of the molecules within the membrane.

These observations suggested that a major portion of the lipid chains in sarcoplasmic reticulum membrane undergoes restricted motion consistent with organization of hydrocarbon chains in a lamellar phase[1]. Insufficient information was obtained, however, regarding the polar ends of the phospholipids molecules. If the phospholipids are assembled in a lamellar phase, one might expect the polar ends to be in a somewhat rigid configuration. In fact, no resolved signal corresponding to choline methyl protons was obtained at 20 °C. On the other hand, this signal was resolved in a spectral line of approx. 20 Hz half width, when the temperature was raised from 20 to 40 °C.

The observed temperature transition is of functional interest in that it is reversible and it correlates with changes of the semipermeability properties of the membrane[1,2]. However, the proton NMR study leaves some uncertainty related to the structural organization of the phospholipid polar ends, since the width of the choline spectral line reflects only intramolecular motion of the choline methyl groups. No information regarding the mobility of the phosphate group was obtained from the proton NMR data.

To further characterize the assembly of phospholipids, we have recorded ^{31}P NMR spectra of intact and experimentally altered sarcoplasmic reticulum membranes, as well as of phospholipids in lamellar and micellar suspensions. The data is used to estimate the mobility of the phosphate head groups and their degree of interaction with protein and other lipids. For comparison, some proton NMR spectra were also recorded.

METHODS

Preparation of membrane fragments

Fragmented sarcoplasmic reticulum was obtained from rabbit leg white muscle. The excised muscle was washed in cold water containing 0.1 mM EDTA (pH 7), and homogenized in 10 mM histidine, 10 % sucrose (pH 7.0). The preparation of sarcoplasmic reticulum was then completed as previously described[1]. The final samples for NMR spectroscopy contained 30–40 mg of membrane phospholipids per ml of 5 mM maleate–^2H_2O (p^{2}H 7). The sarcoplasmic reticulum of these final suspensions was found able to accumulate Ca^{2+} and hydrolyze ATP, as reported elsewhere[2].

Membrane lipids were extracted in chloroform–methanol (2:1, v/v), and washed repeatedly with 100 mM KCl in water[3]. The solvent was evaporated under a stream of nitrogen and the dry residue collected on the walls of a flask. Coarse dispersions of the residue in 5 mM maleate–^2H_2O (p^{2}H 7) were obtained with the aid of glass beads shaken in the flask. Finer suspensions were obtained by sonication.

Measurement of the NMR spectra

The phosphorous NMR spectra were recorded at 101 MHz and a corresponding magnetic field strength of 5.87 Tesla. The spectrometer utilizes a super-conducting solenoid and its design and operation were described elsewhere[4]. The spectra were recorded in a frequency-swept, time-sharing modulation mode and calibrated by

counting the sweep frequency. The signal-to-noise ratio was improved by accumulating repeated scans through the resonance line in a Northern Scientific NS-544 signal averager. Proton NMR spectra were similarly obtained at a spectrometer frequency of 250 MHz with the system "locked" to the residual proton resonance of H^2HO.

The ambient temperature of the sample in the spectrometer is approximately 30 °C. The temperature of the sample could be raised and held at approximately 40 ± 2 °C by heating and regulating the flow of air used to drive the sample spinner turbine. The probe temperature was measured directly with a small thermometer inserted in the sample before and after recording the spectra.

RESULTS

The ^{31}P NMR spectrum of sarcoplasmic reticulum vesicles (Fig. 1a) consists of a single Lorentzian-shaped line of 1.0 ± 0.1 kHz half width and a chemical shift of 8 ± 1 ppm up field from the phosphorus resonance of sonicated dispersions of egg yolk lecithin (Fig. 2a). Equally broad lines are found in unsonicated dispersions of lipid extracted from sarcoplasmic reticulum membranes (Fig. 2b) or those of dipalmitoyl lecithin.

No linewidth narrowing was obtained on addition of chelating agents (0.1 mM EDTA, pH 7). Therefore, the possible broadening effect of paramagnetic metal contaminants was ruled out.

Reduction in the size of the membrane fragments or lipid aggregates by sonication or addition of deoxycholate (30 mM) gives appreciably narrowed lines (Fig. 1b). A line narrowing from 1.0 to 0.75 kHz (half width) is also obtained when the temperature of the sarcoplasmic reticulum vesicles is raised from 30 to 40 °C. As the temperature cools, the line width progressively increases to its original value.

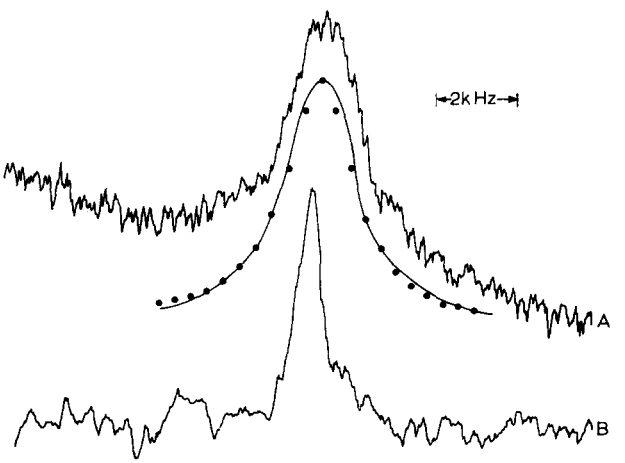

Fig. 1. 101-MHz ^{31}P NMR spectra of (A) sarcoplasmic reticulum membrane fragments (50 mg protein/ml). The smooth curve below the recorded trace is a redrawing to eliminate the base line distortion due to wide sweep widths (approx. 15 kHz). The solid points (●) are computed for a Lorentzian line of 1.05 kHz half width. (B) Sonicated (20 min) sarcoplasmic reticulum membranes (12 mg protein/ml). The half width is approx. 300 Hz. In deoxycholate-treated sarcoplasmic reticulum the half width is approx. 30 Hz.

Denaturing procedures, such as limited trypsin digestion and exposure to temperatures above 45 °C, freezing, or addition of 3 M guanidine, did not significantly change the ^{31}P NMR spectral line obtained from sarcoplasmic reticulum membranes. However, these procedures do change the proton NMR spectra of sarcoplasmic reticulum suspension; doubling the intensity of the resolved choline line, as well as increasing the intensities of lines which may be assigned to protein group protons. (Figs 3a and b).

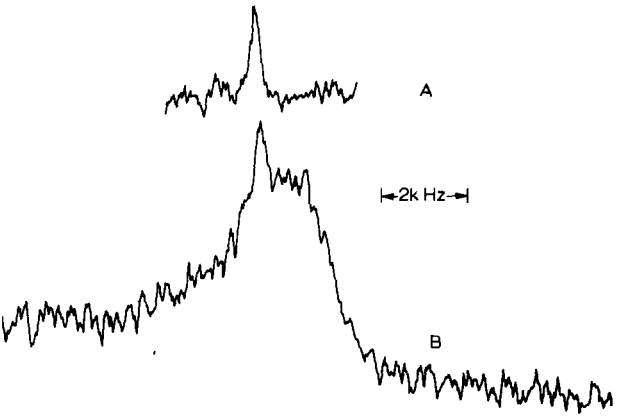

Fig. 2. 101-MHz ^{31}P NMR spectra of (A) egg yolk lecithin micells (approx. 5 mg/ml); (B) a coarse aqueous dispersion of sarcoplasmic reticulum membrane lipids (35 mg/ml) extracted with methanol–chloroform. The scale is the same as in Fig. 1.

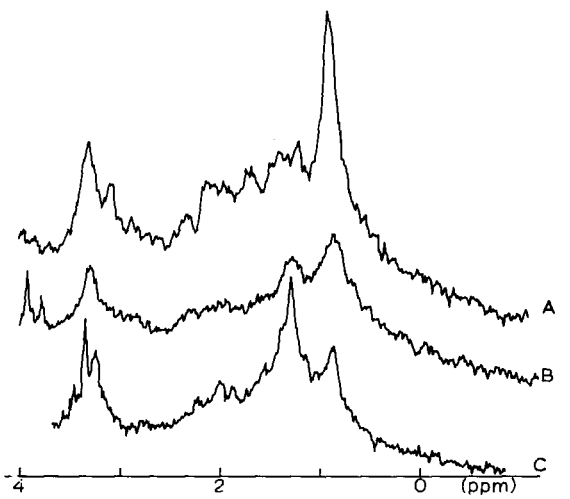

Fig. 3. The 250-MHz ^{1}H NMR spectra of: (A) trypsinized (10 min) and freeze–thawed sarcoplasmic reticulum membranes; (B) intact sarcoplasmic reticulum membrane fragments; (C) lipid extract of sarcoplasmic reticulum membranes. The membrane lipid concentrations as well as the spectrometers settings are the same in A and B. All the samples are suspensions in $^{2}H_2O$ buffered to pH approx. 7.0 with 10 mM maleate. The lipid concentrations are 50 mg/ml. The scale is in ppm from tetramethylsilane (zero point) the lines at 0.9 ppm are assigned to CH_3 groups; those at 1.4 ppm to $-(CH_2)_n-$ groups; and those at 3.3 ppm to the $(CH_3)_3\,N^+-$ groups of choline.

DISCUSSION

The observed half width, $\Delta\nu$ for the phosphorus resonances in sarcoplasmic reticulum and lipid dispersions is roughly half that predicted from the calculated second moment[5],* $\langle\Delta\nu^2\rangle$ which is $4.1 \cdot 10^6$ to $5.7 \cdot 10^6$ Hz^2. If only neighboring proton magnetic dipoles interact with ^{31}P nuclei, the reduction of the ^{31}P line width from the predicted rigid lattice limit may be interpreted in terms of random modulations of the local dipolar fields[6]. The modulation may be accomplished *via* proton spin-lattice relaxation or fluctuations of the internuclear spatial variables. The time-constant or correlation time τ_c^P, characteristic of these fluctuations may be estimated[6] within an order of magnitude to be $\Delta\nu/2\pi\langle\Delta\nu^2\rangle$ or approx. $3 \cdot 10^{-5}$ s. Since proton spin-lattice relaxation times, T_1^H, in lipids are on the order of milliseconds[8,13], a correlation time of 10^{-5} s is more likely to correspond to spatial fluctuations of the internuclear coordinates. Furthermore, the estimated time constant for Brownian rotation of the sarcoplasmic reticulum fragments is long (approx. 10^{-3} s) compared to 10^{-5} s, and indicates that τ_c is characteristic of molecular motion. Compared to the hydrocarbon chains and choline groups, the characteristic motion of this portion of the lipid molecule is thus 10^2–10^3 times slower. Similar considerations apply to the lipid dispersions.

This is not true of sonicated or deoxycholate-treated materials. These procedures reduce the particles to a size which have Brownian motions characteristic of molecular dimensions[8,9]. As originally proposed by Chapman, this rapid rotation of the particles masks slower intramolecular motions and gives narrowed lines. (Figs 1b and 2a).

In addition to line broadening by nuclear magnetic dipole interactions, local diamagnetic (electron) currents might also produce an inhomogeneous distribution of local magnetic fields and broaden the ^{31}P NMR line. Such broadening is field dependent and can be estimated by extrapolating line widths measured at various field strengths to zero external magnetic field[8]. In preliminary study, ^{31}P NMR lines recorded from a coarse aqueous dispersion of egg yolk lecithin at 36.4 MHz (2.11 T) had halfwidths 450 ($\pm$50) Hz. Extrapolation of the sarcoplasmic reticulum and synthetic lipid ^{31}P line widths (measured at 5.87 T) through this point gives a zero field width of approx. 200 Hz, thus τ_c^P ($H_0 \to 0$) approx. $0.6 \cdot 10^{-5}$ s. Accurate estimates of the field dependence of the ^{31}P line widths in membranes are limited by poor signal-to-noise conditions.

While there appears to be some field-dependent broadening of the ^{31}P NMR lines in lipid dispersions ,the amount is not sufficient to significantly alter the esti-

* The equation for the powder average second moment[4] of a resonance line for spin, I, coupled to nonresonant spins S is:

$$4\pi^2\langle\Delta\nu^2\rangle = \frac{4}{15}\gamma_I^2\gamma_S^2\hbar^2 S(S+1)\sum_k 1/r_k^6$$

Where γ_I, γ_S are the gyromagnetic ratios of spins I and S: $\hbar^2\gamma_S^2 S(S+1)$ is the squared magnetic moment of spin S and r_k is the distance between I and the kth spin S. For ^{31}P coupled to protons, $\langle\Delta\nu^2\rangle$ about $4.73 \cdot 10^8 \Sigma (1/r^6)$ Hz^2 when r is given in units of 10^{-8} cm. For phospholipids, a value of r_{P-H} approx. 2.6–2.8 Å (estimated from X-ray diffraction data for glycerylphosphorylcholine[6]) was used for the four nearest neighboring α-carbon protons. More remote protons contribute less than 5% to the second moment.

mated τ_c^P. It is concluded that the motion of the phosphate head group is considerably restricted compared to other portions of the lipid molecule in unsonicated sarcoplasmic reticulum membranes and lipid dispersions. It is also of interest to point out that the correlation time approx. 10^{-5} s derived from the ^{31}P NMR data is in good agreement with recent findings of Kornberg and McConnell[9]. By a totally independent method these authors estimated the jump time for the lateral diffusion of spin-labeled lipid analogues in lecithin micells was less than $3 \cdot 10^{-4}$ s.

Phosphorus NMR signals in sarcoplasmic reticulum membranes appear to be insensitive to protein denaturation. This is to be contrasted with the observed increase in the population of mobile choline groups when sarcoplasmic reticulum membranes are briefly trypsinized and denatured by freezing or heating (Fig. 3a and b). Likewise separation of the lipids from the protein by chloroform–methanol extraction[3] shows an overall increase in the high resolution portions of the lipid's proton NMR spectrum (Fig. 3c), but only minor changes in the ^{31}P spectrum of the lipid extracts (Fig. 2b).

The phosphate and choline groups also differ in their responses to changes in temperature. In sarcoplasmic reticulum a portion of the choline groups are reversibly converted from a bound to a freely rotating state when the temperature is raised. From the width of the choline peak the lifetime in the rotating state is estimated to be $\geqslant 0.3$ s. On the other hand the temperature dependence of the ^{31}P NMR spectrum presents no suggestion of a two-state transition of the type observed for the choline group. The observed 25 % narrowing is entirely consistent with the notion that the ^{31}P NMR line width is determined by modulation of the local dipolar fields. Increasing the temperature increases the rate or frequency of modulation, $1/\tau_c^P$ and thus narrows the line. The data also suggests the mobility of the choline group may be independent of that of the phosphate group.

The results obtained with ^1H and ^{31}P NMR spectroscopy of sarcoplasmic reticulum vesicles suggests that in the native state of the membrane, the majority of phospholipids are assembled with strong immobilization of the polar ends, but greater motional freedom of the aliphatic chains. This is consistant with a lamellar assembly of phospholipids previously proposed by Finean *et al.*[11,12] who obtained wide angle X-ray diffraction patterns with 4.5Å band attributed to alligned hydrocarbon chains. We should point out, however, that the proton NMR data suggests that some fraction of the choline groups and hydrocarbon chains are more immobilized than others, possibly by association with membrane protein. Further experimentation will be required to clarify this point. The mobilities of these groups are indeed more sensitive to protein denaturation than the phosphate group.

After this work was completed and the manuscript was in its final stages of preparation the authors were pleased to read, in the recent communication of Barker *et al.*[14], the results of pulsed 84.5-MHz NMR studies of ^{31}P relaxation times in a variety of lipids. When corrections are made for field-dependent contributions, the reported ^{31}P transverse relaxation time, T_2, in egg lecithin is in excellent agreement with our estimate of T_2 from the ^{31}P NMR line width. Also in accord with our ^{31}P data for membranes is the report of a single, increasing T_2 for lipid ^{31}P nuclei as a function of increasing temperature. These authors note too, that only in phosphatidylserine does the replacement of H_2O with H^2HO cause line narrowing. Accordingly we found no differences in ^{31}P line widths of sarcoplasmic reticulum mem-

branes suspended in H_2O or H^2HO. This is consistant with their results and the low phosphatidylserine content (approx. 10%) of sarcoplasmic reticulum membranes[15].

ACKNOWLEDGEMENTS

The authors wish to thank Professor A. A. Bothner-By for the use of his NMR facilities which are supported by National Institutes of Health Grant RR 00292. We are also grateful to Dr J. Dadok who specially modified the MPC250 spectrometer for high sensitivity operation at 101 MHz and to Dr C. H. Löchmuller for the use of his 36.4-MHz spectrometer.

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