BBA 75689

PROTON NUCLEAR MAGNETIC RESONANCE STUDIES OF SARCOPLASMIC RETICULUM MEMBRANES

CORRELATION OF THE TEMPERATURE-DEPENDENT Ca^{2+} EFFLUX WITH A REVERSIBLE STRUCTURAL TRANSITION*

DONALD G. DAVIS[a] AND GIUSEPPE INESI[b]

[a]*Department of Physiology and Pharmacology, Duke University Medical Center, Durham, N. C. 27706 (U.S.A.) and* [b]*Department of Biochemistry, Carnegie-Mellon University Pittsburgh, 4400 Fifth Avenue, Pittsburgh, Pa. 15213 (U.S.A.)*

(Received March 18th, 1971)

SUMMARY

Proton nuclear magnetic resonance spectra of ATP-dependent Ca^{2+}-accumulating sarcoplasmic reticulum vesicles isolated from rabbit leg white muscle show a reversible, temperature dependent structural transition. This transition involves a transformation of the choline methyl groups of lecithin from a rigid to a mobile state. The energy of the transformation is approx. 35 kcal/mole and involves nearly 60 % of the lecithin molecules in the membrane. The fraction of mobile methyl groups as a function of temperature correlates with the temperature-dependent Ca^{2+} efflux measured in these preparations. As determined by nuclear magnetic resonance, other agents which alter protein structure and simultaneously increase Ca^{2+} efflux also increases the fraction of mobile choline methyl groups. Likewise, irreversible heat denaturation of the membrane protein alters the temperature dependence of the transition.

The proton magnetic resonance spectra are consistent with the organization of approx. 80 % of the lipid fatty acid chains into a lamellar phase which permits only limited motion. On the other hand approx. 20 % of the lipid molecules are in a fluid region of the membrane in which nearly isotropic motion occurs ($\tau_c \approx 10^{-10}$ sec).

INTRODUCTION

Proton nuclear magnetic resonance (NMR) studies on red cell membrane fragments, as well as on lipoproteins and lipid micelles, have indicated the potential usefulness of this technique in determining some molecular aspects of membrane structures[1-3]. In some cases, for example, estimates of the degree of molecular motion and local order can be obtained from NMR spectra, as these two parameters may be

Abbreviation: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

* Reprint requests: Dr. Donald G. Davis, Department of Physiology and Pharmacology, Duke University Medical Center, Durham, N. C. 27706 (U.S.A.)

related to the spectral line widths. Due to differences in chemical shifts, resonance lines characteristic of lipid and some protein groups can be resolved and different membrane structural components studied in appropriate experimental conditions.

Sarcoplasmic reticulum is a very promising system for studies attempting to relate structure and function of membranes. In fact, large amounts of purified vesicular fragments of sarcoplasmic membranes can be isolated from muscle tissues, and functional properties, such as ion transport and permeability, can be conveniently studied. High rates of Ca^{2+} uptake, coupled to ATP hydrolysis, are sustained by the sarcoplasmic reticulum vesicles, indicating a high frequency of active sites. No other major activity is associated with these membranes.

In this report, proton NMR spectra of sarcoplasmic reticulum vesicles are compared to those of pure phospholipid micelles. The effect of experimental procedures known to alter membrane permeability is also reported. Monitoring the activity of these membranes offered an opportunity to check the native state of the structures examined on NMR spectroscopy.

METHODS

Preparation of membrane vesicles

Sarcoplasmic reticulum was prepared by homogenization of rabbit leg white muscle (100 g) in 300 ml of 20 mM histidine, and 10 % sucrose; 20 min centrifugation at 15000 × *g*; 90 min centrifugation of the supernatant at 40000 × *g*; resuspension of the sediment in 10 mM histidine, 0.6 M KCl and 10 % sucrose; incubation for 1 h at 3°; 20 min centrifugation at 15000 × *g*; collection of the supernatant and 90 min centrifugation at 40000 × *g*; resuspension of the sediment buffered (p^{2}H 7) ^2H_2O and 2 h centrifugation at 40000 × *g*. The ^2H_2O washings were repeated 3 times. The final suspension contained 50–70 mg protein per ml. Ca^{2+} accumulation and ATPase activity of this suspension were studied as previously described[4].

In some experiments, previous to washings with ^2H_2O, 100 mg of sarcoplasmic reticulum protein were incubated with 1 mg of trypsin for 10 min at 25°. The incubation was interrupted by the addition of 2 mg of trypsin inhibitor. It was previously demonstrated that this short digestion removes an outer granular layer from the outer surface of the sarcoplasmic reticulum vesicles (electron microscopy of negatively stained preparations). When 10 % of the protein is solubilized, the vesicles 'leak' Ca^{2+}, but a very active ATPase remains associated with the vesicles[4].

Measurement of the NMR spectra

The proton NMR spectra were recorded at 90 MHz on a Bruker HFX-3 NMR spectrometer. The spectrometer system was operated in a frequency swept mode and locked to the strong H^{2}HO signal. At 25° this line is shifted —4.84 ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)*. In some cases, the signal to noise ratio was improved by using a Fabri-Tex Model 1074 time averaging computer. The probe and sample temperatures were controlled *via* the variable temperature accessory provided with the spectrometer. The probe temperature was measured by a

* The chemical shift scale used in this paper assigns a shift of 0 ppm to DSS and assigns negative shifts to lines downfield from DSS.

precalibrated thermocouple placed directly beneath the NMR sample tube. The temperatures are accurate and reproducible to $\pm$ 0.25°

RESULTS

Vesicular fragments of sarcoplasmic reticulum, washed twice in ^2H_2O and stored in a concentrated suspension for 24 h at 2–3°, maintain their ability to accumulate Ca^{2+} with a specific activity comparable to those of previously described preparations[1].

Proton NMR spectra of concentrated (50–60 mg protein per ml) suspensions of sarcoplasmic reticulum show fairly well resolved peaks that can be compared to those of sonicated dispersions of phospholipid (Fig. 1). The main resonance lines at —0.90, —1.26 and —2.00 ppm can be attributed to (CH_3), $-(C\underline{H}_2)_n-$ and $(C\underline{H}_2-CH=C)$ protons, respectively[5]. No spectral line corresponding to choline methyl protons (—3.3 ppm) is obtained at 20°.

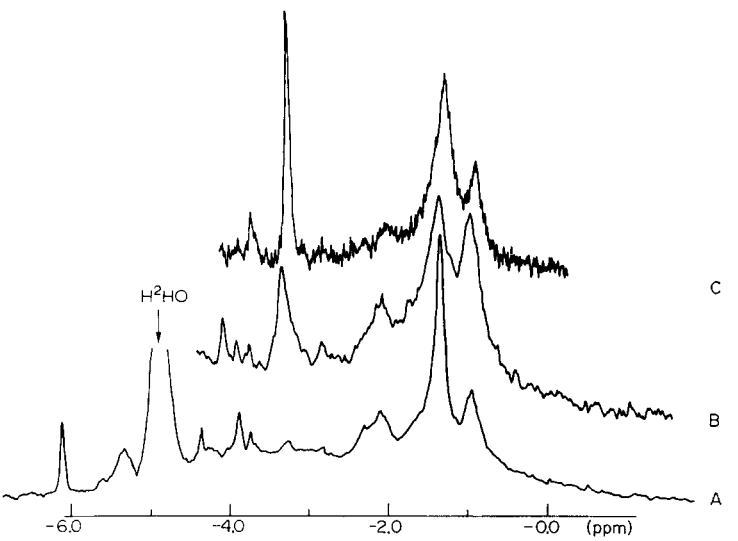

Fig. 1. Portions of the 90-MHz proton NMR spectra of (A) 6 % (protein wt.) sarcoplasmic reticulum suspension in ^2H_2O, (B) 0.6 % sonicated suspension of sarcoplasmic reticulum vesicles and (C) 0.5 % sonicated dispersion of egg lecithin. All records were taken at 25°. Note the appearance of the choline line at $\approx$ —3.3 ppm and the intensity increase of the methyl line at $\approx$ —0.9 ppm in the sonicated sarcoplasmic reticulum suspension.

The total intensity of the signal (Fig. 2) is equal to that of an equivalent amount of phospholipid dissolved in chloroform. The well resolved peaks, however, only correspond to approximately one fifth of the total observed signal. The remaining appears as a broad line of approximately 500 Hz line width.

A 10-fold dilution of the suspension does not change the spectrum, except for a proportional decrease in the intensity of the signal. Sonication of the membrane suspension produces a marked decrease in turbidity, an increase in the intensity of all the well resolved lines of the NMR spectrum, and the appearance of a signal corresponding to choline methyl protons (Fig. 1).

As opposed to sonication, raising the temperature of an sarcoplasmic reticulum suspension produces reversible changes of the NMR spectra, while it does not decrease the turbidity of the sample. As the temperature is raised above 20°, a line corresponding to choline methyl protons appears, and its intensity increases to reach a plateau between 40 and 50° (Fig. 3). Over this temperature range, the line width ($\approx$ 20 Hz) of the signal remains unchanged. A simultaneous increase of the methyl groups signal intensity is also observed.

After this plateau, higher temperatures cause transformation of the suspension into a gelatinous mass, and NMR spectra similar to those obtained with sonicated

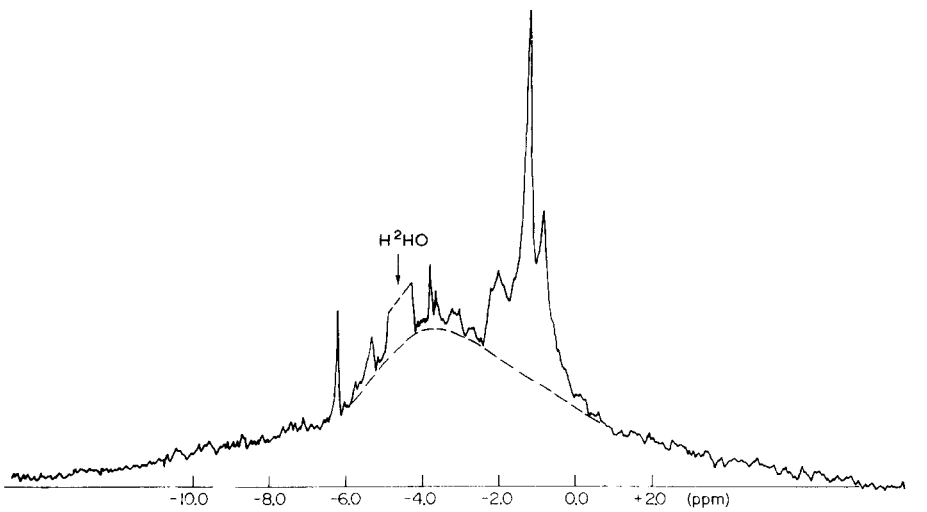

Fig. 2. The 90-MHz proton NMR spectrum of sarcoplasmic reticulum vesicles (60 mg protein per ml ^2H_2O) recorded over a wide sweep range. Note the broad line superimposed upon the high resolution portion of the spectrum. The lines at $\approx$ −4.8 and −6.0 ppm are the protons of H^2HO and 8 mM/maleic acid buffer, respectively.

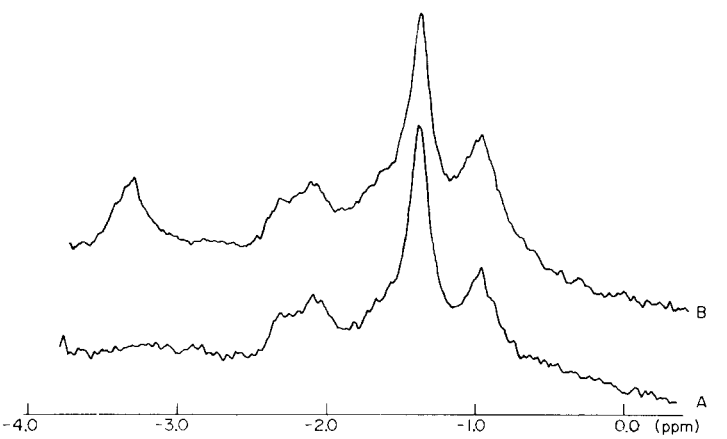

Fig. 3. A portion of the proton NMR spectra of sarcoplasmic reticulum vesicles at two temperatures. Record A: 20°. Record B: 40°. Note the new line at $\approx$ −3.3 ppm due to mobile $(CH_3)_3\,N^+$– groups, and the intensity increase in the high held CH_3– resonance at $\approx$ −0.9 ppm.

sarcoplasmic reticulum. In sarcoplasmic reticulum exposed to temperature higher than 50°, an apparent shift of the 'melting' curve is observed (Fig. 4a), and the plateau between 40 and 50° is abolished.

Spectra similar to those of native sarcoplasmic reticulum at 40° were obtained with trypsin-treated sarcoplasmic reticulum, even at 25°.

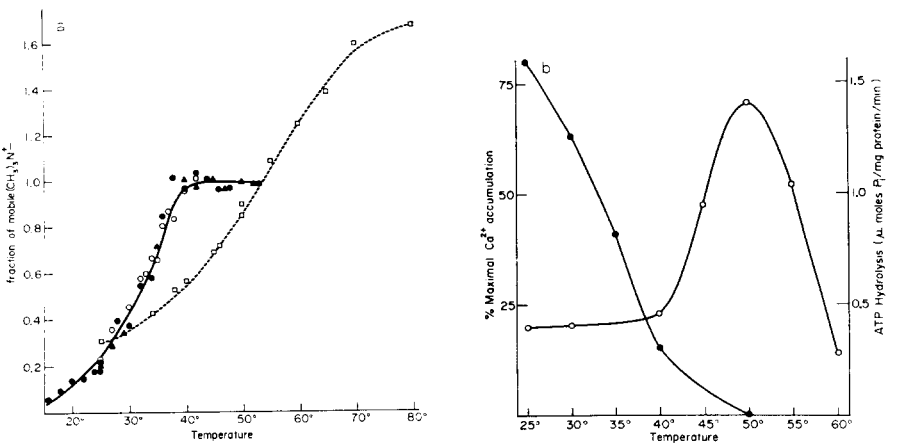

Fig. 4. (a) The temperature dependence of the choline proton NMR signal intensity. The ratio of the intensity of the choline methyl resonance line at different temperatures to the intensity of this line at 40°–42°, $I_{°C}/I_{42°}$ is plotted on the ordinate. ● and ▲, temperature of the preparations (two) changed from low to high (15 to 50°); ○, same preparation as ● but showing the reverse temperature course, high to low; □, same preparation as ▲ but the temperature was first raised to ≈ 80° and then lowered to room temperature. Note that the plateau at 40 to 50° is abolished after exposure of the preparation to temperatures in excess of 50°. (b) Functional properties of sarcoplasmic reticulum vesicles at different temperatures. ●, % Ca^{2+} retention; ○, ATPase activity. These values are taken from the paper of JOHNSON AND INESI[19].

DISCUSSION

At 25°, the major portion of the NMR spectrum of sarcoplasmic reticulum vesicles has a line width of ≈ 500 Hz. This value is only 1 % of the 'rigid lattice' line width found in phospholipids at —196° (ref. 6) and indicates narrowing by thermal motion[7]. Since sarcoplasmic reticulum vesicles are 0.25 μm or larger in diameter, the estimated time constant for Brownian motion of the particles is too slow (approx. 1 sec) to influence the NMR line width[7]. Therefore, the powder average is the appropriate distribution of signal intensity and thermal motion of the molecules within the membrane determines the observed line width[6,7].

Analysis of steady state NMR line width in particulate systems must be made with some care when direct measurements of the spin-lattice and spin-spin relaxation times (T_1 and T_2, respectively) are not available[9–11]. In this situation, however, some limits on the rate and type of molecular motion can be established.

A crude estimate of the correlation time[7], τ_c, for the thermal motion of these molecules is given by

$$\Delta\nu_{obs} \simeq 2\pi\tau_c \langle\Delta\nu^2\rangle \qquad (1)$$

where $\langle\Delta\nu^2\rangle$, $\Delta\nu_{obs}$ and τ_c are the rigid lattice second moment, the width of the

narrowed line and the molecular correlation time, respectively. CHAPMAN AND SALSBURY[6] found $\Delta\nu^2$ to be approx. $3 \cdot 10^9$ Hz^2 for phospholipids at —196°. From this value and our line width we estimate τ_c to be approx. 10^{-6} to 10^{-8} sec·rad^{-1}. This calculation is only approximate and may underestimate the true rate of molecular motion. Residual line broadening may be produced by anisotropic motion of the lipid fatty acid chains and incomplete averaging of local magnetic field inhomogeneities[9–11]. Our results are consistent with a major portion of the lipid molecules moving in a restricted, partially ordered way as in a lamellar phase. In fact, the intensity of the resolved methylene protons increased when sarcoplasmic reticulum was sonicated to form a clear micellar dispersion. No like increase was observed in sarcoplasmic reticulum treated with protein disrupting agents, trypsin or 8 M urea.

On the other hand, approximately 15–20 % of the NMR signal of sarcoplasmic reticulum is well resolved, indicating rapid, isotropic motion ($\tau_c \lesssim 10^{-9}$ sec·rad^{-1}) of a disordered, liquid-like fraction of aliphatic chains in these membranes. The relatively large signals at —2.0 ppm ($\underline{CH_2}CH=$) and —5.4 ppm ($-\underline{CH}=C$) suggest that the high resolution portion of the NMR spectra is due to unsaturated fatty acid chains present in sarcoplasmic reticulum[12]. Such a low viscosity region in sarcoplasmic reticulum membranes was also suggested by HUBBEL AND MCCONNELL[13], based on the ESR line shape of the spin label, tetramethylpiperidine-1-oxyl, partitioned in the membrane.

It cannot be said with certainty that this fluid-like lipid fraction is a constituent of the native membrane. The altered intensities and line widths in NMR spectra of sarcoplasmic reticulum preparations (Fig. 1B) deliberately fragmented by sonication, as well as the fairly uniform appearance and size distribution of the sarcoplasmic reticulum particles[4] suggest that fluidization is not the result of fragmentation damage to the particles.

A most interesting feature of the spectra obtained at 20° is the absence of a well resolved resonance signal deriving from choline methyl protons. As the temperature is raised, however, a resonance line corresponding to these choline methyl protons appears at —3.3 ppm. The sigmoidal nature of the intensity increase with temperature and the constancy of the resonance linewidth suggests a type of cooperative transition in which the fraction of rotating choline groups depends on temperature as well as the number of molecules in the mobile state. From the resonance line width, the lifetime of molecules in the mobile state is at least 0.3 sec or longer.

No simultaneous change in methylene proton signal is observed. This is consistent with mobilization of lipid molecules in a lamellar array, since the fatty acid protons are restricted to motion about the long axis of the chain and thus still subject to homogeneous broadening by anisotropic local magnetic fields[6,11].

If, as it is often assumed for the reversible, thermal denaturation of proteins[14–16], the equilibrium between the rigid and mobile lipid fractions is described simply as the ratio between these two fractions:

$$K_{eq} = \frac{f_{mobile}}{1 - f_{mobile}} \qquad (2)$$

the slope of the curve log K_{eq} *vs.* T^{-1} (Fig. 5) yields, formally, an estimate of the van 't Hoff heat, ΔH_{vH} for the transition. This data gives a value of 35 ± 5 kcal/mole and is comparable to that value derived by MELCHIOR *et al.*[17] from differential calori-

metry studies of *Mycoplasma laidlawii* membrane preparations in which similar thermal transitions are thought to occur.

The results reported here also indicate this thermal transition involves protein as well as lipid molecules. Trypsinization of the sarcoplasmic reticulum suspension results in an increase in the choline methyl intensity at 25° is equivalent to that in the untreated preparation at 37°. After heat denaturation of the protein at temperatures above 50°, the intensity–temperature profile follows the course indicated in Fig. 4a. The plateau between 40 and 50° is abolished and from 50 to 80° the choline methyl line intensity increases another 80%. This suggest that the nature of the transition and the fraction of phospholecithin molecules involved in the transition is controlled by the integrity of the protein structure and lipid-protein interactions.

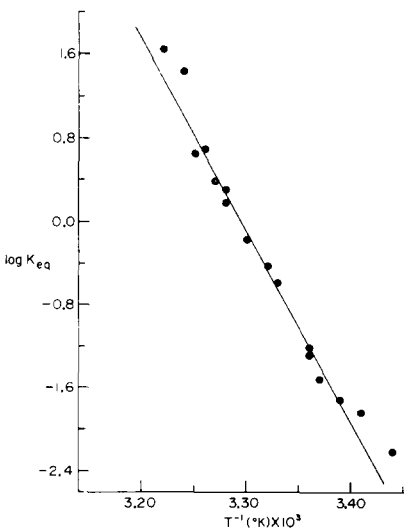

Fig. 5. Change in the apparent equilibrium constant (K_{eq}) for the reversible transition of choline methyl groups from a rigid to mobile configuration. From the slope of the line, $\Delta \ln K/\Delta T^{-1}$, a van 't Hoff heat, ΔH_{vH}, of $\approx$ 35 kcal/mole is calculated.

An interesting comparison can be made between the effect of temperature on the proton NMR spectra and some functional parameters of the sarcoplasmic reticulum vesicles, such as the ability to retain accumulated Ca^{2+} and ATPase activity (Fig. 4b). The resonance signal of the choline methyl protons and the ability of the sarcoplasmic reticulum vesicles to retain accumulated Ca^{2+}, is correlated in the first temperature transition. A relation between semipermeability properties and structural changes in sarcoplasmic reticulum was previously proposed, based on experiments with spin labels[18]. Parallel to the second temperature transition, irreversible ATPase inactivation is produced, as a consequence of protein denaturation.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Chien Ho for making his NMR facilities available to us, and to Miss Kam Yu, for preparing our sonicated lipid dispersions.

REFERENCES

1 D. CHAPMAN AND S. A. PENKETT, *Nature*, 211 (1966) 5055.
2 D. CHAPMAN, V. B. KAMAT, J. DE GIER AND S. A. PENKETT, *Nature*, 213 (1967) 74.
3 D. CHAPMAN, V. B. KAMAT, J. DE GIER AND S. A. PENKETT, *J. Mol. Biol.*, 31 (1968) 101.
4 G. INESI AND H. ASAI, *Arch. Biochem. Biophys.*, 126 (1968) 469.
5 D. CHAPMAN AND A. MORRISON, *J. Biol. Chem.*, 241 (1966) 5044.
6 D. CHAPMAN AND N. J. SALSBURY, *Trans. Faraday Soc.*, 62 (1966) 2607.
7 E. R. ANDREW, *Nuclear Magnetic Resonance*, Cambridge Univesity Press, London, 1955, p. 165.
8 A. ABRAGAM, *The Principles of Nuclear Magnetism*, Oxford University Press, London, 1961.
9 J. R. HANSEN AND K. D. LAWSON, *Nature*, 225 (1970) 542.
10 B. SHEARD, *Nature*, 223 (1970) 1057.
11 S. KAUFMAN, J. M. STEIM AND J. H. GIBBS, *Nature*, 225 (1970) 743.
12 K. WAKU AND Y. NAKAZAWA, *J. Biochem. Tokyo*, 56 (1969) 95.
13 L. W. HUBBEL AND H. M. MCCONNELL, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 12.
14 A. J. SOPHIANOPOULOS AND B. J. WEISS, *Biochemistry*, 3 (1964) 1920.
15 C. C. MCDONALD, W. D. PHILLIPS AND J. D. GLICKSON, *J. Am. Chem. Soc.*, 93 (1971) 235.
16 J. M. O'REILLY AND F. E. KARASZ, *Biopolymers*, 9 (1970) 1429.
17 D. L. MELCHIOR, H. J. MOROWITZ, J. M. STURTEVANT AND T. Y. TSONG, *Biochim. Biophys. Acta*, 219 (1970) 114.
18 G. INESI AND W. LANGRAF, *J. Bioenergetics*, 1 (1970) 355.
19 P. N. JOHNSON AND G. INESI, *J. Pharmacol. Exptl. Therap.*, 169 (1969) 308.