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Two Populations of Phospholipids Exist in Sarcoplasmic Reticulum and in Recombined Membranes Containing Ca-ATPase†

Barry S. Selinsky and Philip L. Yeagle*

ABSTRACT: Phosphorus nuclear magnetic resonance spectra of sarcoplasmic reticulum membranes from rabbit muscle and of recombined membranes containing the calcium-dependent adenosinetriphosphatase (Ca-ATPase) of sarcoplasmic reticulum reveal two distinguishable, overlapping resonances. One resonance resembles a normal phospholipid bilayer resonance, and the other is much broader. The broader component is not seen in protein-free phospholipid vesicles. In recombined membranes of the Ca-ATPase, the intensity found in the broad component was proportional to the concentration of protein in the vesicles. The two-component spectra are interpreted to arise from at least two different domains of phospholipids, one of which is motionally restricted by the Ca-ATPase. Phospholipids exchange between these two domains at a rate less than 10^3 s^{-1}. A model for protein–lipid interactions in membranes containing the Ca-ATPase is proposed in which some of the phospholipid head groups of the membrane interact directly with the protein.

Since the proposal of the fluid-mosaic model for membrane structure (Singer, 1974), much effort has been expended to determine how membrane lipids interact with proteins embedded in the lipid bilayer. Previous studies measured the effect of membrane proteins on spin-labeled or deuterated lipid probes located in the hydrophobic interior of the phospholipid bilayer. The results obtained were dependent upon the exact location and composition of the probe, and the time scale of the measurement. Little information is available on the behavior of the membrane surface in the presence of membrane proteins.

Sarcoplasmic reticulum isolated from rabbit skeletal muscle is an ideal system in which to study protein–lipid interactions. Over 90% of the protein of the light fraction of sarcoplasmic reticulum is the calcium-dependent adenosinetriphosphatase (Ca-ATPase)[1] (Meissner, 1975). The structure and function of this transmembrane pump have been extensively studied (Tada et al., 1978; Ikemoto, 1982). Furthermore, the Ca-ATPase can be easily solubilized in detergents and examined free of lipids (Dean & Tanford, 1978) or reconstituted into a defined lipid environment (Bennett et al., 1978a).

In a previous study (Albert et al., 1981), we measured interactions between phospholipids and the Ca-ATPase protein of sarcoplasmic reticulum by using phosphorus NMR. Phosphorus NMR has several advantages over deuterium NMR and electron spin resonance (ESR) in the study of protein–lipid interactions. First, phosphorus-31 is 100% naturally abundant, and a foreign probe does not have to be introduced. Second, phosphorus NMR measures the properties of the phospholipid head groups which likely are important to biological membrane function (Yeagle, 1978; Seelig, 1978). In our earlier study, using an indirect method (Albert et al., 1981), some of the phospholipid head groups in a crude

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[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; Ca-ATPase, calcium-dependent adenosinetriphosphatase; FID, free induction decay of the ^{31}P NMR signal; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CSA, chemical shift anisotropy.

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preparation of sarcoplasmic reticulum were shown to be closely interacting with the Ca-ATPase.

Using different instrumentation and a different method of data collection, we can now directly visualize a phospholipid head-group domain which is motionally restricted relative to the normal phospholipid bilayer in these membranes. In this paper, we observe such domains in purified fractions from sarcoplasmic reticulum and in reconstituted membranes.

Materials and Methods

Egg phosphatidylcholine was purchased from Avanti Polar Lipids, Inc., Birmingham, AL. This phospholipid produced a single spot when analyzed by two-dimensional thin-layer chromatography with the following solvent systems: (a) chloroform–methanol–ammonia (65:25:5); (b) chloroform–acetone–methanol–acetic acid–water (6:8:2:2:1) (Rouser et al., 1970). The plates were developed by sulfuric acid charring. Phosphoenolpyruvate, ATP, ADP, pyruvate kinase, lactic dehydrogenase, NADH, and arsenazo III were purchased from Sigma Chemical Co., St. Louis, MO. Sodium deoxycholate and octyl glucopyranoside were purchased from Calbiochem-Behring Corp., American Hoechst, San Diego, CA.

Preparation of Sarcoplasmic Reticulum. Sarcoplasmic reticulum was isolated from white hind leg muscles of New Zealand White rabbits as previously described (Eletr & Inesi, 1972). The crude isolate was further purified into light and heavy fractions by discontinuous sucrose density gradient centrifugation (Fernandez et al., 1980). Sarcoplasmic reticulum samples were stored in 20 mM HEPES, 100 mM KCl, and 1 M sucrose, pH 7.0, under argon at 4 °C. NMR measurements were performed on these membrane samples without further preparation. Measurements on native membranes were made within 2 days of preparation. Material used for the lipid extracts and reconstitution experiments was frozen and stored in the same buffer at −70 °C until ready for use.

Assays. Phospholipid concentrations were determined by the Bartlett (1959) method as modified by Litman (1973). Protein concentration was determined in the presence of SDS by using the method described by Lowry et al. (1951) with bovine serum albumin as the standard. Discontinuous polyacrylamide gel electrophoresis was done in the presence of SDS (Laemmli, 1970) as modified by Smith et al. (1975). The 10% polyacrylamide gels were stained with Coomassie blue stain and scanned at 550 nm.

ATPase activity was assayed at 37 °C in the presence of 0.1 mM calcium chloride to obtain Ca-dependent activity and in 1.0 mM EGTA to obtain Ca-independent activity (Warren et al., 1974). The assay medium including 10 mM KH_2PO_4, 5 mM Mg, and 100 mM KCl, pH 7.0. Calcium transport was measured in the same medium plus 50 μM arsenazo III, a calcium-sensitive dye (Herbette et al., 1977). Typical ATP hydrolysis activities were 1.0–2.0 μmol (mg of protein)$^{-1}$ min^{-1} for light sarcoplasmic reticulum and 0.5–2.0 μmol (mg of protein)$^{-1}$ min^{-1} for heavy sarcoplasmic reticulum. Calcium transport efficiency was typically 0.1–0.5 calcium transported/ATP hydrolyzed for light sarcoplasmic reticulum and 0.1–0.3 calcium transported/ATP hydrolyzed for the heavy fraction. With 20 mM HEPES and 10 mM potassium oxalate as a precipitating agent, the calcium transported/ATP hydrolyzed ratio for light sarcoplasmic reticulum increased to 1.2–1.6, which is comparable to other preparations (Meissner, 1975). We chose phosphate buffer as the common buffer for our activity measurements because oxalate interferes with the coupled ATP hydrolysis assay, and it was important to measure ATP hydrolysis and calcium transport under the same conditions.

We measured ATP hydrolysis activity and calcium transport activity before and after NMR measurements to ensure that the measurement conditions do not adversely affect the membrane preparation. In all cases, at least 90% of the enzymatic activity was retained after NMR measurements.

Phase-Separated Sarcoplasmic Reticulum. For one experiment, light sarcoplasmic reticulum was incubated at 37 °C for 72 h. The sample was purged with argon before incubation to prevent lipid oxidation. At the end of the incubation, the sample had formed a large gellike aggregate. Preliminary studies indicated that the aggregation was due to phase separation initiated in the membrane vesicles (B. S. Selinsky, A. Sen, S. W. Hui, and P. L. Yeagle, unpublished experiments). The aggregated material was sonicated in a bath sonicator for two 10-s bursts, applied to a continuous 0–50% sucrose density gradient, and centrifuged for 12 h at 130000g at 4 °C. Two bands appeared on the gradient, a thin band with a phospholipid/protein ratio of 200/1 and a dense band with a phospholipid/protein ratio of 50/1. The latter band was harvested and concentrated by centrifugation at 200000g at 4 °C for 30 min. The final pellet was suspended in 20 mM HEPES, 100 mM KCl, and 1 M sucrose, pH 7.0., for measurements using ^{31}P NMR.

Preparation of Lipid Extracts from Sarcoplasmic Reticulum. Total lipid extracts of light sarcoplasmic reticulum were prepared by the Folch (1957) method. The aqueous phase included 1 mM EGTA to chelate free calcium. The extracted lipids were dried first under nitrogen and then briefly under vacuum and suspended without sonication in 20 mM HEPES, 100 mM KCl, 1 M sucrose, and 1 mM EGTA, pH 7.0, for NMR studies.

Reconstitution of the Ca-ATPase. Recombined membranes containing the Ca-ATPase were prepared by first solubilizing light sarcoplasmic reticulum in deoxycholate and precipitating the ATPase with poly(ethylene glycol) (Dean & Tanford, 1977). The delipidated protein precipitate was resuspended in 1.0 mL of 20 mM HEPES, 0.1 M KCl, and 1.0 mM dithiothreitol, pH 7.5, containing 20 mg of octyl glucopyranoside. Egg phosphatidylcholine was solubilized in 2.0 mL of the same buffer, added to the protein suspension, and vortexed for 30 s. This mixture was then diluted 1:5 into the same buffer except without detergent and layered onto a discontinuous 15%/50% sucrose gradient. The gradient was centrifuged at 130000g for 6 h. The turbid band at the 15%/50% interface was isolated and concentrated by centrifugation at 200000g at 4 °C for 30 min. The pellet was suspended in 20 mM HEPES, 100 mM KCl, and 1 M sucrose, pH 7.0, and stored under argon at 4 °C for NMR measurements. These recombined membranes have a high ATP hydrolysis activity [4–10 μmol (mg of protein)$^{-1}$ min^{-1}] but do not accumulate calcium. Other investigators (Knowles & Racker, 1975; Moore et al., 1981) have been unable to reconstitute calcium transport activity in phosphatidylcholine recombined membranes. When a similar procedure is used, transport function is not reconstituted into vesicles containing phosphatidylcholine and cytochrome oxidase (Madden et al., 1983).

Electron Microscopy. Electron micrographs were prepared on a Siemons 101 electron microscope at 80 kV. Sarcoplasmic reticulum samples were stained with 2% ammonium molybdate directly on Formvar carbon-coated grids.

Nuclear Magnetic Resonance. ^{31}P nuclear magnetic resonance (NMR) spectra were obtained at 109 MHz with a JEOL FX270 Fourier transform spectrometer on a broad-band probe in 10-mm tubes at 20 °C. A fully phase cycled (32-pulse) chemical shift anisotropy (CSA) echo was used with

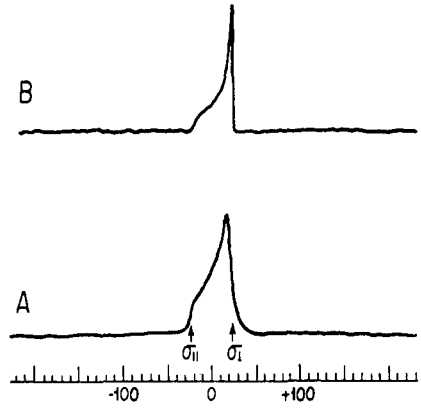

FIGURE 1: 109-MHz ^{31}P NMR spectra of a multilamellar dispersion of egg phosphatidylcholine, suspended in 20 mM HEPES, 100 mM KCl, and 1 M sucrose, pH 7.0, taken in 10-mm tubes at 20 °C by using the echo technique described in the text with exponential filtering of (A) 800 and (B) 50 Hz. A total of 2000 scans were recorded on 30 mg of phospholipid. The scale values are in units of parts per million.

a 20-μs echo. The CSA echo sequence eliminates base-line artifacts, removing the need for first-order phase corrections (Rance & Byrd, 1983). Furthermore, pairs of 70° (10-μs), 140° pulses were used to more adequately excite the full frequency range of the observed resonances, as previously suggested was feasible (Rance & Byrd, 1983). Proton decoupling, gated on only during acquisition to minimize sample heating and to inhibit the buildup of a nuclear Overhauser effect (Yeagle et al., 1975), was employed. A decoupling field of 9 kHz was used. A spectral width of 50 kHz was used, and is displayed in the figures; 2048 data points were collected with a 1-s repetition time. T_1 was measured by using the inversion recovery method with a homospoil, and T_2 was measured by using the Carr–Purcell–Meiboom–Gill pulse sequence.

Spectral Simulations. Spectral simulations were calculated on a computer by using the equations of Seelig (1978). In this procedure, the transition probability for an axially symmetric powder pattern is convoluted with Lorentzian line shapes to produce the spectrum. Spectral simulations were fit to the original spectra by eye. The spectra displayed in Figure 6 were also fit by mathematically comparing the experimental spectra in digital form with their corresponding simulations, also in digital form, minimizing the following function:

$$R = \sum|\text{obsd}_i - \text{sim}_i|/\text{obsd}_i$$

where R = the fitting factor, obsd_i = the digital value of the observed spectra at point i, and sim_i = the digital value of the simulated spectra at point i. During this fit, the relative intensity of the two components was varied, as well as the effective chemical shift anisotropy and line width. This fitting process resulted in simulated spectra virtually identical with those simulations fit by eye. In simulating the broad feature found in some of our ^{31}P NMR spectra, either a broad Lorentzian line or a broad trapezoidal line shape was used, with similar results.

Results

^{31}P NMR spectra from phospholipids in membrane vesicles and large unilamellar or multilamellar phospholipid vesicles exhibit a partially motionally averaged powder pattern (Figure 1). These spectra show the undistorted powder pattern obtained by using the echo technique (Rance & Byrd, 1983). We used both large (800 Hz) and small (50 Hz) exponential filters on our data prior to Fourier transformation for signal

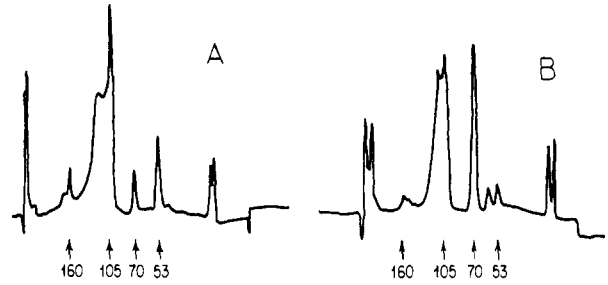

FIGURE 2: SDS-polyacrylamide gel electrophoresis of sarcoplasmic reticulum membranes. Densitometric scans for (A) light sarcoplasmic reticulum and (B) heavy sarcoplasmic reticulum. The molecular masses indicated (in kilodaltons) were determined by simultaneous determination of standard protein molecular weight markers.

enhancement. Scans A and B of Figure 1 are the same egg phosphatidylcholine spectra processed with an 800- and a 50-Hz filter, respectively. The added filtering distorts the line shape somewhat but does not affect the base line. Also, large unilamellar vesicles, prepared as described previously (Mimms et al., 1981), exhibit the same line shape as multilamellar dispersions (data not shown). This is an important control, since electron microscopy data show that the sarcoplasmic reticulum vesicles and recombined membranes described later are unilamellar (Inesi & Scales, 1974).

Characterization of Sarcoplasmic Reticulum. Figure 2A shows SDS–polyacrylamide gel electrophoresis of light sarcoplasmic reticulum, which contains a large band at 105 kdalton (the Ca-ATPase). Also seen are a 70-kdalton band representing calsequestrin and bands at 160 and 53 kdalton characteristic of the two glycoproteins found in this system (Campbell & MacLennan, 1981). The heavy fraction of sarcoplasmic reticulum has a protein composition similar to that of light sarcoplasmic reticulum but contains a higher concentration of calsequestrin (Figure 2B), as reported previously (Meissner, 1975). Also, the acyl chains of the phospholipids of the heavy fraction are more unsaturated than the light fraction (Van Winkle et al., 1982). Negative-stain electron micrographs of light sarcoplasmic reticulum vesicles (not shown) demonstrate that the vesicles have diameters between 400 and 2000 Å.

^{31}P NMR of Light Sarcoplasmic Reticulum. Figure 3 shows the ^{31}P NMR spectra of several different lipid and sarcoplasmic reticulum samples. Figure 3B shows the spectrum of an unsonicated dispersion of the total lipid extract prepared from sarcoplasmic reticulum. A pseudoaxially symmetric powder pattern is obtained. Figure 3C shows the ^{31}P NMR spectrum of light sarcoplasmic reticulum. The majority of the resonance is similar in shape to that reported previously for unfractionated sarcoplasmic reticulum (Albert et al., 1981). A pseudoaxially symmetric powder pattern is seen.

However, the resonance shape is more complex than that observed for a pure phospholipid bilayer. First, the resonance is more triangular, losing some of the definition attributed to a pseudoaxially symmetric powder pattern. This broadening of the spectrum is due to a shortened spin–spin relaxation time (T_2) (Rice et al., 1979). As seen in Table I, the T_2 for native sarcoplasmic reticulum phospholipids is 10 times shorter than that for egg phosphatidylcholine. Second, the maximum has shifted slightly downfield with respect to the pure lipid samples, due to a reduction in the effective chemical shift anisotropy ($\Delta\sigma$).

There is a third, more significant, difference between the sarcoplasmic reticulum spectrum and the spectra of pure phospholipids. Careful examination of the edges of the powder

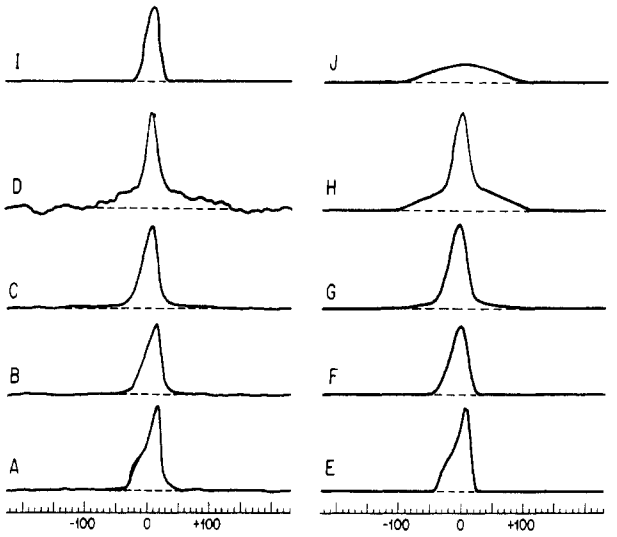

FIGURE 3: 109-MHz ^{31}P NMR spectra, taken as described in Figure 1, of (A) egg phosphatidylcholine, (B) lipids extracted from sarcoplasmic reticulum, (C) light sarcoplasmic reticulum, and (D) lipid-depleted light sarcoplasmic reticulum. All membrane samples were measured in 20 mM HEPES, 100 mM KCl, and 1 M sucrose, pH 7.0. Spectra E, F, G, and H are the spectral simulations of spectra A, B, C, and D, respectively, calculated as described in the text. Spectra I and J represent the shape of the two components which make up spectrum H (not to the same scale).

Table I: Relaxation Parameters of Sarcoplasmic Reticulum and Lipids Extracted from Sarcoplasmic Reticulum

sample	T_1 (s) (±10%)	T_2 (ms) (±10%)
light sarcoplasmic reticulum	1.5	1.7
heavy sarcoplasmic reticulum	1.3	1.7
lipids extracted from sarcoplasmic reticulum	0.7	6.8
egg phosphatidylcholine	1.4	14

pattern reveals that the resonance, for the biological membrane, does not return sharply to the base line as it does in the phospholipid liposome or vesicle spectra but extends out on either side for 150–200 ppm on the chemical shift scale before returning to the base line. This broad feature can best be observed by using a 50-kHz spectral window, a CSA echo sequence, and a high signal/noise ratio.

The broad feature thus far has only been observed in protein-containing membranes. It is therefore reasonable to hypothesize that membrane protein may have some influence on the ^{31}P NMR resonance shape. To test this hypothesis, it would be desirable to increase the protein content of the membrane. We have devised a method to lower the lipid to protein ratio in native light sarcoplasmic reticulum by a factor of 2 (see Materials and Methods). The ^{31}P NMR spectrum of this lipid-depleted light sarcoplasmic reticulum is shown in Figure 3D. The broad feature is much more pronounced in this sample than in the normal sarcoplasmic reticulum.

Spectral Simulations for Light Sarcoplasmic Reticulum. To more adequately understand these spectra, it is necessary to simulate the spectra by using the appropriate theoretical expressions. We have done so by using the equations of Seelig (1978). $\sigma_\perp$ and $\sigma_\parallel$ were estimated from the experimental spectra. It is also necessary to obtain T_2 for these simulations to use in the equation for the Lorentzian line shape. In Table I are reported the T_2 values, measured at $\sigma_\perp$, for the systems described above. Using average T_2 values provided adequate simulations. It should be noted that T_2 is anisotropic across the powder pattern for these systems (Rice et al., 1979) and

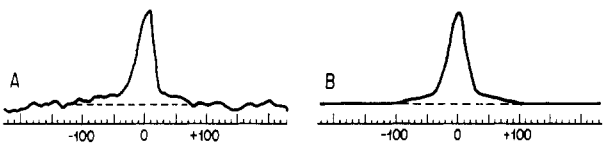

FIGURE 4: 109-MHz ^{31}P NMR spectrum taken as described in Figure 1 of (A) heavy sarcoplasmic reticulum, suspended in 20 mM HEPES, 100 mM KCl, and 1 M sucrose, pH 7.0. (B) Spectral simulation of spectrum A, taken as described in the text.

reflects predominantly the "normal bilayer" feature of the spectra. Use of an average T_2 value does not significantly affect the conclusions of this work. Corrections for exponential filtering were also included.

Figure 3E,F shows that by using the simulation method described above, the ^{31}P NMR spectrum of egg phosphatidylcholine and lipids extracted from sarcoplasmic reticulum can be satisfactorily simulated. Using this method for the light sarcoplasmic reticulum produces the simulation seen in Figure 3I. This simulation takes into account a somewhat more complete motional averaging of the ^{31}P chemical shift tensor due to the observed vesicle size distribution. Although this simulates well the "normal bilayer" portion of the biological membrane spectrum, the broad feature described earlier is not simulated. Clearly, a simulation using the assumption of a homogeneous phospholipid head-group environment, as the above simulation does, will not produce the broad feature of the experimental spectrum.

To adequately simulate the experimental data, it is necessary to add a second, much broader component to the simulations. As a first approximation, a broad Lorentzian line was used. This is not because the broad feature in the experimental spectra is expected to be Lorentzian (which in fact predicts an incorrect value of the isotropic chemical shift) but because at the definition of the spectra achieved experimentally, a more analytical simulation would not produce a visibly or mathematically better fit. The broad resonance used is seen in Figure 3J at an arbitrary intensity. The simulated spectra in Figure 3I,J were then added, with different relative intensities, to produce the results in Figure 3G,H. As can be seen, by using two components, the experimental data of Figure 3C,D can be successfully simulated.

The most important feature of these simulations is that the broad component of these spectra cannot be simulated by assuming only one resonance. To accurately simulate the experimental spectra, it must be assumed that the lipid in both the light sarcoplasmic reticulum and the lipid-depleted light sarcoplasmic reticulum exists in two domains. These two domains give rise to two overlapping resonances. Furthermore, two-component spectra are evident only in membranes containing protein, and most prominently the Ca-ATPase.

We can calculate from these simulations the percent of the total phospholipid in each of the two domains. Furthermore, these domain populations can be normalized with the molar ratio of the phospholipid to Ca-ATPase content in the membranes. These data are reported in Table II. It is evident that the population of the domain characterized by the broad component in the native membrane is approximately equal to that in the lipid-depleted membrane when normalized.

^{31}P NMR of Heavy Sarcoplasmic Reticulum. The ^{31}P NMR spectrum of heavy sarcoplasmic reticulum vesicles appears in Figure 4A. It appears similar to that of the native light sarcoplasmic reticulum in Figure 3C, including the presence of a significant broad feature. The spectral simulation of these data appears in Figure 4B. It can only be simulated by adding a second, broad component. The number of

Table II: Number of Phospholipids Motionally Restricted by Ca-ATPase in Sarcoplasmic Reticulum and in Recombined Systems[a]

sample	no. of samples measured	lipid: Ca-ATPase	lipids restricted	
			Ca-ATPase[b]	corr[c]
light sarcoplasmic reticulum	4	118:1	27	12
heavy sarcoplasmic reticulum	2	118:1	50	24
Ca-ATPase recombined with phosphatidylcholine	1	138:1	50	24
	1	84:1	31	14
	1	83:1	41	18
	1	130:1	56	26
av of recombined membranes	4		45	21
lipid-depleted light sarcoplasmic reticulum	1	50:1	27	12

[a] The calculations employed to determine the number of phospholipids motionally restricted and the corrected number of phospholipids motionally restricted can be found in the text. [b] The number of phospholipids motionally restricted per Ca-ATPase content in the membrane, as described in the text. [c] The corrected value for the number of phospholipids motionally restricted per Ca-ATPase. The criteria used to determine these values are described under Discussion.

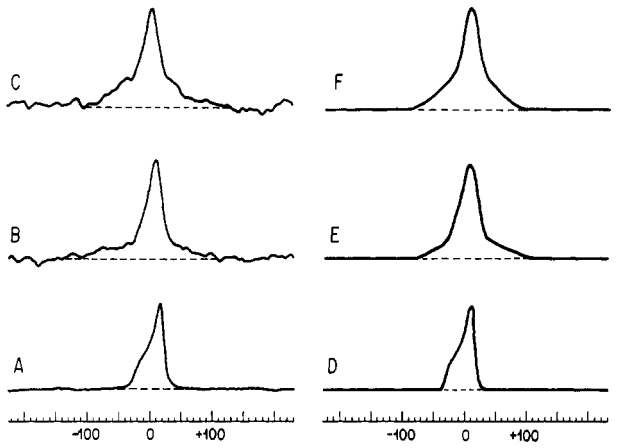

FIGURE 5: 109-MHz ^{31}P NMR spectra of (A) egg phosphatidylcholine, (B) Ca-ATPase recombined with egg phosphatidylcholine at a lipid to protein ratio (molar) of 130:1, and (C) Ca-ATPase recombined with egg phosphatidylcholine at a lipid to protein ratio of 80:1. The preparation of the recombined membranes is described in the text. Spectra D, E, and F are the spectral simulations of spectra A, B, and C, respectively, as described in the text.

phospholipids in the domain characterized by the broad component, normalized for Ca-ATPase content, is reported in Table II.

As a control for all the above measurements, ATP hydrolysis and calcium transport were measured on all-protein-containing samples before and after NMR experiments. In all cases, less than 10% of the enzymatic activity of the Ca-ATPase was lost during the NMR measurement.

^{31}P NMR of Reconstituted Ca-ATPase. From the sarcoplasmic reticulum data, a reasonable suggestion is that the Ca-ATPase may be inducing the phospholipids of the membrane to form two domains. This can be tested by placing purified Ca-ATPase in a defined lipid environment. Figure 5 shows spectra of Ca-ATPase and egg phosphatidylcholine reconstituted membranes at two different phospholipid to protein ratios. The normal bilayer component of these spectra closely resembles previously reported spectra (Rajan et al., 1981). However, the techniques used in this study show that a broad feature is also present in these spectra. Figure 5 shows the spectral simulations for the experimental data. As in the case of the native membranes, the experimental data cannot be simulated without the addition of a second, much broader component. As the number of phospholipids per protein decreases, the intensity of the broad component increases, and a greater percentage of the total membrane phospholipid is found in this domain. Again, this can be normalized for the content of Ca-ATPase in the membrane. Table II shows the results for the recombined membranes whose ^{31}P NMR spectra are displayed in Figure 5 and for two other recombined membranes with similar phospholipid to protein ratios. When normalized for protein content, a reasonably constant population for the broad domain is found.

Discussion

Rapid axial rotation of the phosphate ^{31}P chemical shift tensor partially motionally averages the 220 ppm anisotropic chemical shift tensor (Kohler & Klein, 1977). The result is a pseudoaxially symmetric powder pattern with a residual chemical shift anisotropy of 45 ppm (Griffin, 1976; Kohler & Klein, 1977) as seen in Figure 1. It has been experimentally determined (Seelig, 1978) that the right edge of the powder pattern represents the chemical shift when the normal to the membrane surface is perpendicular to the external magnetic field. The left edge of the powder pattern corresponds to a parallel orientation of the normal to the membrane surface and the external magnetic field. The remaining orientations give rise to the rest of the powder pattern. The pseudoaxial symmetry induced by axial rotation of the phospholipid head group allows the complete description of the powder pattern by just $\sigma_\perp$ and $\sigma_\parallel$.

The ^{31}P NMR spectra of sarcoplasmic reticulum and the membrane fractions derived from that membrane also show a pseudoaxially symmetric powder pattern. This is consistent with the phospholipid bilayer felt to be common to each of the membranes. In addition, comparison of the ^{31}P NMR spectra from protein-free lipid dispersions with the ^{31}P NMR spectra from the intact, functional biological membrane reveals a broad spectral feature in the sarcoplasmic reticulum spectrum, in addition to the normal bilayer resonance.

Several possible explanations for this broad feature must be considered. First, the broad feature is not attributable to an artifact for the following reasons. The free induction decay (FID) of this sample reproducibly shows a fast-decaying second component which is not visible in the FIDs of pure phospholipid dispersions. We have observed the broad spectral feature in samples prepared from 10 different rabbits. Spectra of protein-free phospholipid membranes taken at the same time on the same instrument under identical conditions do not exhibit this broad feature. The addition of non-sarcoplasmic reticulum protein to phospholipids does not in and of itself produce a broad component. Obtaining more than 10 times as many scans of a weak phospholipid sample does not reveal any intrinsic instrumental artifacts or base-line distortions (Albert & Yeagle, 1983).

Another explanation of this broad spectral feature that needs to be considered is incomplete ^{1}H decoupling. The static H–P

dipolar interaction for a phosphodiester (with no directly bonded protons) is about 6 kHz (Chan et al., 1981). The strength of the decoupler on the instrument used was measured to be 9 kHz. Therefore, incomplete decoupling is not likely a cause of the broad feature. Also, we have obtained spectra of dry phospholipids. The resultant powder pattern is 220 ppm wide on the chemical shift scale (Griffin, 1976). The edges of the powder pattern return sharply to the base line, indicating that any residual dipolar broadening is small compared to the full chemical shift tensor.

In addition, if the broad feature is a residual from a partially decoupled system, then with no decoupling there should be even more intensity outside the normal bilayer region. Instead, the experimental result was that the broad feature became more difficult to see without decoupling, attributable to dipolar broadening which spreads the intensity over an even wider range. This is the result expected if the broad feature is a true spectral component. Another test of the hypothesis is to examine the system with even higher decoupling power. A previous study, which also shows evidence for broad ^{31}P NMR spectral features from protein-containing membranes as discussed below, reports a higher decoupler power than used here (Rajan et al., 1981). Thus, the results from a variation of decoupler power are inconsistent with the hypothesis that the broad feature is due to incomplete ^{1}H decoupling.

Another hypothesis for the broad feature is that at some orientation of the membrane the H–P dipolar interaction is too strong and the spin–spin relaxation too fast to decouple effectively. Two experimental observations rule out this hypothesis. One is that although the $T_{1\rho}$ of the protons in this system (on the millisecond time scale) is short, it is not sufficiently short to cause this problem (P. L. Yeagle and J. Frye, unpublished results). Second, it is known, as described above, that each position in the powder pattern corresponds to a particular orientation of the membrane. If a specific orientation is not decoupled, but has its intensity spread out in the broad feature, then there should be a corresponding hole in the powder pattern representing that orientation. The size of the hole should reflect the magnitude of the broad component. Careful comparison of the spectrum for the native membrane, Figure 3C, with that of the total lipid extract, Figure 3B, shows that no such hole exists.

The best explanation of the data is that at least two separate domains of phospholipids exist in native sarcoplasmic reticulum membranes and in Ca-ATPase recombined membranes. Spectral simulations show that the experimental data can only be successfully simulated by adding two quite different spectral components. No simulation using a single homogeneous phospholipid environment can reproduce the experimental data. Also, the domain characterized by the broad component is protein dependent. An increase in protein content leads to an increase in the intensity of the broad component. In the absence of protein, no broad component is seen.

The broad feature observed here is most likely attributable to a domain of phospholipids whose head groups are motionally restricted compared to protein-free membranes. The width of the anisotropic ^{31}P NMR spectrum of hydrated phospholipids is normally around 45 ppm (Seelig, 1978), representing a partially motionally averaged system. If the rate or freedom of motion is restricted, then the ability to motionally average the chemical shift tensor may be reduced or eliminated. This would increase $\Delta\sigma$ and therefore broaden the resonance associated with the restricted phospholipids.

One can theoretically predict a resonance width for a phospholipid bound to the Ca-ATPase in the sarcoplasmic reticulum. We begin with the analysis of Campbell et al. (1979). This analysis focuses on two motions capable of averaging the anisotropic chemical shift tensor of the phospholipid phosphate. One motion is the overall motion of the membrane vesicle. The distribution of vesicle sizes determined by electron microscopy suggests some motional averaging of the ^{31}P chemical shift tensor may result from tumbling of the sarcoplasmic reticulum vesicles. An estimate of this contribution is obtained by comparison of the residual chemical shift anisotropy observed for the sarcoplasmic reticulum vesicles (38 ppm) to that observed from unsonicated dispersions of phosphatidylcholine (45 ppm), whose multilamellar liposomes rotate too slowly to cause significant motional averaging. Therefore, this contribution is less than 10 ppm.

The other motion that contributes to motional averaging of the chemical shift tensor is rotation of the phospholipid phosphate while bound to the protein. [This motion is important because the off rate of phospholipid head groups from protein sites is slow compared to protein rotation (see below).] This motion is controlled by protein rotation with a correlation time for the Ca-ATPase in the range of 60–200 μs (Thomas & Hidalgo, 1978; Kirino et al., 1978). Using the analysis of Campbell et al. (1979) predicts that this rotation will cause a reduction in the observed chemical shift anisotropy of 60 ppm or less. Therefore, the total contribution to motional averaging from these two motions is 70 ppm or less, resulting in a residual chemical shift anisotropy for phospholipid bound to the Ca-ATPase in the membrane of 150 ppm or greater. The experimental data indicate an observed chemical shift anisotropy for the broad component of 150–200 ppm, in excellent agreement with the theoretical prediction.

The two domains must be in slow exchange with one another on the NMR time scale. If fast exchange were occurring, we would not see a two-component spectrum but a one-component spectrum which would represent the weighted time average of the spectrum derived from each domain. Also, we would not see two components in the FID of protein-containing membranes if exchange were fast. Under the conditions used in these measurements, a rate of exchange between the two domains of 10^3 s^{-1} or faster would result in a time-averaged powder pattern. Since two distinguishable components are observed, the exchange rate must be slower than that limit.

An important question is why such a broad component has not been seen in most previous ^{31}P NMR spectra of membranes. In the conventional NMR experiment, excitation occurs by a 90° pulse, followed by a period of dead time and data collection. If the T_2 of the broad feature is very short, then the phosphorus atom may relax too quickly to be observed in the normal Fourier transform NMR spectrum. Also, if the resonance is very broad, then the decay of the free induction decay (T_2^*) will occur too quickly to see fully under the same conditions. This fast relaxation is a likely explanation for why two domains have not been observed in most other studies in the past. We are using a spin-echo technique which allows us to begin measurement immediately after the refocusing of the echo. The broad component is seen clearly under these conditions. We find that if we increase our dead time, the intensity of the broad component is rapidly reduced. Further, the CSA echo sequence used here is fully phase cycled which removes many of the base-line artifacts which would otherwise be a problem.

Substantial evidence exists which supports this two-domain model. Although this work represents the first clearly defined report of two-component NMR spectra for intact sarcoplasmic reticulum membranes, two-component NMR spectra can be

observed in two recent reports (Rajan et al., 1981; Seelig et al., 1981) which cannot be simulated by using a one-component model. Furthermore, two lipid domains are indicated by reconstitution experiments (Bennett et al., 1978a), phospholipase digestion (Bennett et al., 1978b), electron spin resonance (Hesketh et al., 1976; Thomas et al., 1982), calorimetry (Lentz et al., 1983), and carbon-13 NMR of the *N*-methyl groups of phosphatidylcholine (Robinson et al., 1972). In all of the above measurements, 20–30 phospholipids have been identified as interacting with each Ca-ATPase. This represents remarkable quantitative agreement among a wide variety of techniques and independent laboratories. Furthermore, the carbon-13 NMR experiments require exchange rates (between domains) as slow as that indicated here for the ^{31}P NMR measurements.

Results obtained by using ^{2}H NMR are not inconsistent with our two-domain model. Seelig et al. (1981) recently described experiments where ^{2}H NMR measurements were performed on recombined membranes of Ca-ATPase. They suggested a 1-MHz rate for exchange from sites next to the protein to sites not immediately adjacent to the protein. This interpretation referred to the position of their label, which was at the 16 position on one fatty acyl chain. This assumes that all the phospholipid domains in the membrane were represented in the spectra (see below). We propose that the interaction between phospholipid and protein occurs at the phosphate group. This does not necessarily require similar motional restriction of the acyl chains. A Ca-ATPase-bound head group can act as a pivot about which the acyl chain can access both restricted and relatively nonrestricted environments (Andersen et al., 1981). This same hypothesis was suggested to explain ^{2}H NMR results on recombined membranes containing cytochrome oxidase (Kang et al., 1979).

^{2}H NMR data may not show two-component spectra because of the correlation times involved. A lipid interacting with a membrane protein would be expected to adopt motions characteristic of the protein. Cross-polarization ^{13}C magic angle spinning NMR of protein-containing membranes shows no resonance attributable to protein (Sefcik et al., 1983). Since protein concentration is not the major problem, protein motion must be. From the protein rotational correlation times referred to above, it is clear that the spectral density of the proton–proton dipolar interaction on the protein carries considerable intensity at or near the frequency corresponding to the proton decoupler power. This leads to a short protein proton $T_{1\rho}$ and the nonobservance of ^{13}C cross-polarization resonances. Therefore, lipids bound to the protein must have some intense motional fluctuations at frequencies in the 10–100-kHz range. This is of the order of the ^{2}H quadrupole coupling and would lead to a loss of signal from deuterated lipids in such an environment.

The number of phospholipids per Ca-ATPase in the broad component reported in Table II is greater than numbers previously reported for sarcoplasmic reticulum and recombined membranes of the Ca-ATPase (Albert et al., 1981). The NMR results reported here were obtained by using a 1-s delay between scans. The phospholipids in the bulk bilayer of all of the membrane systems studied here have a T_1 of approximately 1.5 s (Table I). This means that under the conditions of our measurements 55% of the resonance of the narrow component was saturated. If we assume that the T_1 of the broad component is short [which has been observed in membranes of high protein content (A. D. Albert, B. S. Selinsky, and P. L. Yeagle, unpublished experiments)] (<0.3 s) relative to the narrow component, we can correct for this saturation. With the correction, our numbers agree well both with previous studies and with data to be presented in a forthcoming paper. Values employing these corrections also appear in Table II.

We have demonstrated that at least two domains of phospholipid exist in sarcoplasmic reticulum membranes and in recombined membranes of the Ca-ATPase isolated from the same system. These data do not limit the model to only two domains, however. Two is the smallest number of domains that is consistent with our data.

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Interactions of a Phenothiazine Tranquilizer with Phosphatidylcholine and Phosphatidylcholine/Cholesterol Membranes†

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ABSTRACT: The interaction of the phenothiazine type tranquilizer methochlorpromazine (MCP) with phosphatidylcholine and phosphatidylcholine/cholesterol bilayer membranes has been studied by ^2H and ^{31}P nuclear magnetic resonance. The effect on bilayer order was monitored with the use of both deuterium-labeled dipalmitoylphosphatidylcholine and dioleoylphosphatidylcholine. In the absence of cholesterol, MCP is largely intercalated into the upper region of the fatty acyl chains and effectively disorders only the lower portion of the bilayer near the center of the membrane. Cholesterol affects the distribution of the drug among the two regions of phospholipid binding, expelling it to the membrane surface. Cholesterol opposes the disordering of the membrane, as well as expelling the drug, and therefore, the order of the membrane depends on the relative concentrations of the stabilizer, cholesterol, and the destabilizer, methochlorpromazine.

Chlorpromazine is a major tranquilizer as well as, in a broader sense, a general anesthetic (Seeman, 1972). It has been suggested that the pharmacological mode of action of the phenothiazine type drugs is related to their presence in or at biological membranes (Guth & Spirtes, 1964; Bhise et al., 1983). In particular, the reduction in membrane permeability to dopamine has been cited as the origin of their antipsychotic function (Bhise et al., 1983; Creese et al., 1976). The specific interactions responsible may be quite complex and involve disordering of the lipid (Pang et al., 1980), disruption of ion channels (Lee, 1976), interaction with membrane protein (LaBella, 1981), or modification of the membrane surface (Bhise et al., 1983; Zografi & Munshi, 1970; Seeman & Bialy, 1963). Some evidence has been found for the incorporation of chlorpromazine into the hydrophobic region of model membranes (Frenzel et al., 1978; Kitamura et al., 1981), and we have recently reported the presence of two lipid binding sites for methochlorpromazine (MCP)[1] (Forrest & Mattai, 1983). The phenothiazine is displaced from one of these sites by cholesterol. The effect of these agents on membrane organization has been the subject of a number of electron spin resonance studies, some of which explored the effect of cholesterol addition or depletion (Neal et al., 1976; Pang & Miller, 1978; Ogiso et al., 1981). In light of the inability of nitroxide-labeled fatty acid probes to even qualitatively monitor the effect of cholesterol on fluid bilayer systems (Taylor & Smith, 1980), the effect of MCP on the membrane was studied by the nonperturbing methods of ^2H and ^{31}P NMR using both saturated and unsaturated labeled phospholipids. The effects on the membrane components of the presence of cholesterol

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[1] Abbreviations: MCP, methochlorpromazine (2-chloro-10-[3-(trimethylammonio)propyl]phenothiazine); NMR, nuclear magnetic resonance; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; PC, phosphatidylcholine; CHOL, cholesterol; CSA, chemical shift anisotropy; ESR, electron spin resonance; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine.

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