EVIDENCE FOR THE INFLUENCE OF THE PROTEIN-PHOSPHOLIPID INTERFACE ON SARCOPLASMIC RETICULUM Ca^{++} Mg^{++} ATPASE ACTIVITY

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ABSTRACT Sarcoplasmic reticulum from the white hind leg muscle of the rabbit was examined with ^{31}P nuclear magnetic resonance as a nonperturbing probe of phospholipid-protein interactions in the intact membrane. The phospholipids of the sarcoplasmic reticulum appear to inhabit two distinct environments: one very similar in behavior to pure phospholipid lamellar dispersions and the other immobilized by the protein in the membrane. Measurement of the population of the latter environment suggests that it is dependent on salt concentration and probably not due to the Ca^{++} Mg^{++} ATPase of the sarcoplasmic reticulum. This immobilization can be removed completely by papain proteolysis of the membrane protein, but only partially by trypsin treatment. The phospholipid composition of recombinants with the Ca^{++} Mg^{++} ATPase was varied in order to look for effects of the phospholipid-protein interface on enzymatic activity of the Ca^{++} Mg^{++} ATPase. Both transphosphatidylated phosphatidylethanolamine (from egg phosphatidylcholine) and bovine brain phosphatidylserine readily partitioned into the putative boundary layer, whereas under the same conditions soybean phosphatidylethanolamine was excluded. Only phosphatidylserine affected the activity of the enzyme, causing an inhibition that was proportional to the phosphatidylserine content, relative to phosphatidylcholine.

INTRODUCTION

The phospholipid-protein interface in cellular membranes constitutes a pivotal element of membrane structure. In the contemporary fluid mosaic model of biological membranes (1), the question is left open whether membrane proteins exist in a sea of lipid as pure protein entities or as lipid-protein complexes. Consideration of the latter concept provokes questions concerning the effects of the lipid on the protein, particularly if the protein is an enzyme. For example, lipids might serve as modulators of enzymatic activity. A lipid-protein complex might also play a role in transmembrane phospholipid asymmetry, as is seen in membranes like the erythrocyte membrane (2) or the sarcoplasmic reticulum membrane (3, 4).

The sarcoplasmic reticulum of muscle has provided much information on the behavior of a functioning membrane enzyme system. The Ca^{++} Mg^{++} ATPase of the sarcoplasmic reticulum actively pumps Ca^{++} across the membrane at the expense of adenosine triphos-

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phate (ATP) in the presence of Mg^{++} and is the predominant membrane protein of the sarcoplasmic reticulum (5, 6).

Several laboratories have shown that phospholipid is an important component for proper enzymatic function of the Ca^{++} Mg^{++} ATPase (7), and that several synthetic phosphatidylcholines can be substituted for endogenous membrane phospholipid (8–10). Interestingly, detergent is also capable of activating the ATPase (11).

Spin-label and calorimetric measurements have suggested that the enzyme maintains a special layer of phospholipid at the lipid-protein interface with an appellation of annulus or boundary lipid (12–15). But physical measurements have not yet been made on the sarcoplasmic reticulum that have detected such a special environment without the addition of probes or the use of recombined membranes.

^{31}P nuclear magnetic resonance (NMR) is proving to be a powerful, nonperturbing approach to the study of membrane structure (16, 17). Because of the 100% natural abundance of the NMR-sensitive ^{31}P isotope, no probes need to be added to the system. The native phosphorus in the headgroup reports primarily on the condition of the membrane surface, with little ambiguity as to the location of the probe, owing to its known position in the chemical structure of the phospholipid. Protein-induced phospholipid immobilization has been detected in vesicular stomatitus viral membranes (18), with glycophorin from the human erythrocyte membrane (19, 20), and in human low density lipoproteins (21), a procedure that exploits the advantages of the ^{31}P NMR approach.

In this study, ^{31}P NMR data on intact sarcoplasmic reticulum suggest the presence of a phospholipid component in the membrane that is immobilized by the proteins of the membrane. Effects on enzymatic activity on reconstitution with some phospholipid classes were also recorded.

MATERIALS AND METHODS

Egg phosphatidylcholine, bovine brain phosphatidylserine, soybean phosphatidylethanolamine, and transphosphatidylated phosphatidylethanolamine (from egg phosphatidylcholine) were purchased from Avanti Biochemicals, Inc., Birmingham, Ala. They produced a single spot when analyzed with two-dimensional thin-layer chromatography: (*a*) chloroform-methanol-ammonia (65:25:5) and (*b*) chloroform-acetone-methanol-acetic acid-water (6:8:2:2:1) (22). The plates were developed by sulfuric acid charring. ATP, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, papain, trypsin, soybean trypsin inhibitor, and NADH were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium deoxycholate was purchased from Calbiochem-Behring Corp., American Hoechst, San Diego, Calif., and sodium dodecyl sulfate (SDS) from Bio-Rad Laboratories, Richmond, Calif.

Rabbit sarcoplasmic reticulum was isolated from the white hind leg muscles of more than 20 New Zealand white rabbits according to the procedure of Eletr and Inesi (23) for these experiments. Measurements were normally made within 2 d of preparation. If longer storage was required samples were frozen in 0.3 M sucrose. ATPase activity was assayed in the presence and absence of 0.1 mM Ca^{++} at 37°C (8). Assay medium included 100 mM triethanolamine, pH 7.2, 5 mM Mg^{++}, 0.1 mM KCl. 1 mM EGTA was used to obtain the ATPase activity independent of Ca^{++}.

Phosphate concentrations were obtained by the procedure of Bartlett (24) and protein was assayed according to Lowry et al. (25).

Exogenous phospholipids were substituted into the immediate environment of the ATPase by published methods (9). 30 mg each of membrane protein (still in the sarcoplasmic reticulum membrane), deoxycholate, and phospholipid were mixed for 2 h at 4°C in 1 M KCl, 0.3 M sucrose, 10 mM dithiothreitol, 50 mM Tris-Cl, pH 8.0. This material was layered on a discontinuous sucrose

gradient of 15 and 50% sucrose. The gradients were centrifuged overnight at 130,000 *g*. A turbid band at the lower interface was harvested, diluted in 50 mM Tris-Cl, pH 7.0, and centrifuged at 100,000 *g* for 1 h. The pellet was taken up in 0.3 M sucrose, 20 mM Tris-Cl (pH 7), assayed, and analyzed.

The phospholipid composition of the recombinants was determined. The lipids were obtained with a chloroform-methanol-water extraction and separated on two-dimensional thin-layer chromatography, according to the procedure described above. The plates were developed first with ninhydrin to identify phosphatidylserine and phosphatidylethanolamine, and then by sulfuric-acid charring. The spots corresponding to the various phospholipids were then scraped and phosphate analysis performed on the scrapings to determine the phospholipid composition.

^{31}P NMR spectra were obtained on a Bruker WP-200 Fourier transform spectrometer (Bruker Instruments, Inc., Billerica, Mass.) at 81 MHz and 30°C unless otherwise stated, in flat bottom tubes. All the samples were kept in ice until the measurement was to be taken, at which time they were warmed to the stated temperature before measurements were begun. Spectra were obtained with gated broadband proton decoupling (1 W) to remove the ^{31}P [^{1}H] nuclear Overhauser effect (26). Full 90° pulses (11 μs) separated by an interval of 5 × T_1 (spin-lattice relaxation time) were used to insure complete relaxation. Data acquisition began 5 μs after the pulse, with a 50-kHz spectral width. Sample volumes (0.8 ml) were identical in the sample and in the standards, and all of the sample was contained within the receiver coil to insure accurate relative intensities. The same sample tube was used for membrane preparations and standards. Samples were prepared for the NMR experiments by centrifugation of the membraneous material, followed by vortexing of the pellet in the specified buffer and introduction into the sample tube along with D_2O (25% in final sample) for a spectrometer lock signal. Unsonicated egg phosphatidylcholine and total lipid extracts of the sarcoplasmic reticulum were used for the standards, since these dispersions have linewidths similar to the resonance from the sarcoplasmic reticulum. This precaution is necessary because, when the procedures described here are followed, a broad resonance is instrumentally attenuated relative to a narrow resonance. Using an internal HPO_4^- resonance as a standard has two complications, in addition to that just described. First, the sample and standard resonances will overlap, which makes separation of the intensity for the two resonances difficult. Second, a small but variable amount of isotropic resonance frequently shows up in ^{31}P NMR of membrane preparations, which contributes to the same portion of the spectrum as the inorganic phosphate and introduces further error in determination of the standard intensity. Spectral intensities were evaluated by cutting and weighing photocopies of the spectra. Multiple measurements indicated that a variation of <5% in total intensity was seen when this procedure was followed. Spin-lattice relaxation times (T_1) were obtained by the inversion-recovery sequence, with delay times between the 180°-τ-90° pulse sequence of 5 × T_1.

Some spectra were also obtained at 40 MHz on a Varian XL-100 (Varian Associates, Inc., Palo Alto, Calif.) with a Nicolet fourier transform modification. Similar results were obtained.

RESULTS

^{31}P NMR of Sarcoplasmic Reticulum Membranes

The ^{31}P NMR spectra of phospholipids in membranes have a distinctive appearance. The spectra are anisotropic in shape because they represent only partial motional averaging of the anisotropic chemical shift tensor of the phosphorus nucleus in the phosphate of the phospholipids by rapid anisotropic rotation about an axis approximately perpendicular to the surface of the membrane.

Fig. 1 displays the ^{31}P NMR spectrum of an unsonicated dispersion of egg phosphatidylcholine, showing the effective ^{31}P chemical shift anisotropy not averaged by the rotation of the phospholipids in the membrane. No further averaging takes place because the size of these multilamellar liposomes precludes isotropic rotation at a rate that approximates the width of the ^{31}P resonance. Fig. 1 also displays the ^{31}P NMR spectrum of the sarcoplasmic reticulum

membrane. This spectrum closely resembles that of pure egg phosphatidylcholine, which is consistent with the presence of the phospholipid lamellar structure in both membranes. Similar ^{31}P NMR spectra are obtained for the human erythrocyte membrane (27), the chromaffin granule membrane (27), vesicular stomatitus viral membrane (18), *Acholeplasma laidawaii* membrane (28), and the rod outer segment disk membrane (29).

Also shown in Fig. 1 is the ^{31}P NMR spectrum of an unsonicated aqueous dispersion of the lipid extract obtained from the sarcoplasmic reticulum membrane by a chloroform-methanol extraction. This spectrum looks very much like the ^{31}P NMR spectrum of the intact sarcoplasmic reticulum and the unsonicated egg phosphatidylcholine. Because of the similarity in the shapes of the above ^{31}P NMR spectra, one can conclude that the motional averaging of the phospholipid headgroup is similar to that expected because of the bilayer form of the phospholipids in all these membranes. The small isotropic component noted in Fig. 1 *C* is variable, owing to small vesicles, and not characteristic of any one preparation.

Phospholipid Immobilization

Although nothing very surprising has been noted in the shapes of the above spectra, interesting information does appear when the ^{31}P NMR spectra are analyzed in another way. The intensity of the ^{31}P NMR resonance for the membrane preparations was measured as described earlier. A representative plot of the intensity of the ^{31}P NMR spectra for the unsonicated lipid standard dispersions as a function of phospholipid concentration appears in Fig. 2. The linear relationship is observed for total lipid extracts of the sarcoplasmic reticulum and for egg phosphatidylcholine dispersions. When the intensity of the ^{31}P NMR resonance of the sarcoplasmic reticulum is compared with standards, a portion of the phospholipid is found not to contribute to the resonance observed. Presumably, this results because that phospho-

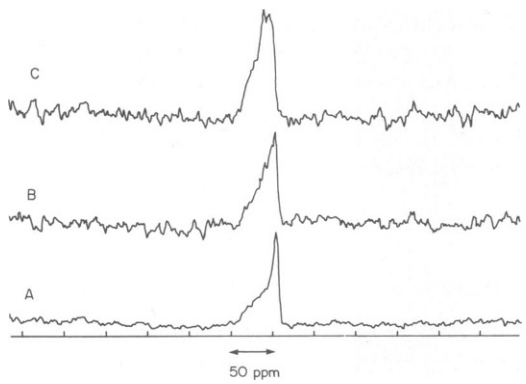

FIGURE 1

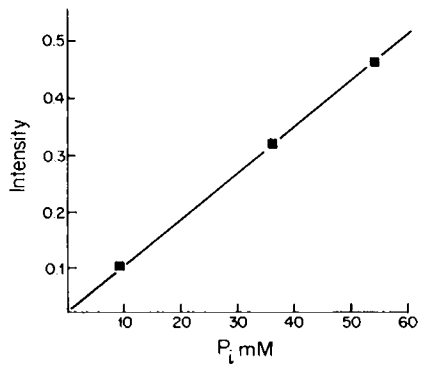

FIGURE 2

FIGURE 1 81 MHz ^{31}P NMR spectra of membrane preparations at 30°C, with a 50-kHz spectral width, 2-K data points, 400 scans with a 7-s repetition rate, and gated broadband ^{1}H decoupling in 10-mm tubes. (*A*) unsonicated egg phosphatidylcholine (38 mM) in 100 mM NaCl, 10 mM Tris, pH 7.6. (*B*) unsonicated $CHCl_3$-CH_3OH extract of sarcoplasmic reticulum in 100 mM NaCl, 10 mM Tris, pH 7.6. (*C*) sarcoplasmic reticulum in 100 mM NaCl, 10 mM Tris, pH 7.6.

FIGURE 2 Intensity of the phospholipid ^{31}P NMR resonance, obtained as described in text, plotted as a function of the phospholipid concentration for unsonicated dispersions of the total lipid extract of the sarcoplasmic reticulum. Intensity units are arbitrary.

TABLE I
PERCENTAGE OF SARCOPLASMIC RETICULUM PHOSPHOLIPIDS IMMOBILIZED

Preparation	No. of animals	Lipids immobilized
		(%)
Normal SR *(no NaCl)*	5	32 ± 1
Normal SR *(100 mM NaCl)*	6	10 ± 2
Trypsin-treated SR	2	21 ± 5
Papain-treated SR	7	0 ± 3
Normal SR *(100 mM KCl, 300 mM sucrose)*	2	0 ± 2
Recombined SR *(100 mM NaCl)*	2	0 ± 4

lipid inhabits an environment in the sarcoplasmic reticulum, which produces a resonance so broad that it does not have substantial intensity in the region of the spectrum where the normal lamellar resonance is observed.

^{31}P NMR intensity data obtained from sarcoplasmic reticulum isolated from several animals appear in Table I. A consistent percentage of the total phospholipid of the membrane fails to contribute to the ^{31}P resonance intensity observed. The average value is 32 ± 1% of the total phospholipid in the membrane in the presence of 10 mM Tris, but no NaCl. Interestingly, a different value is obtained when measured in the presence of 100 mM NaCl (see Table I). These data most likely reflect phospholipid that is in an immobilized environment in the membrane created by the membrane protein of the sarcoplasmic reticulum. The effect of temperature on this immobilized component was measured between 30 and 45°C for 1-yr-old animals. These data are presented in Fig. 3 in the absence of NaCl. A switch to 100 mM KCl, 300 mM sucrose produced another interesting effect. As shown in

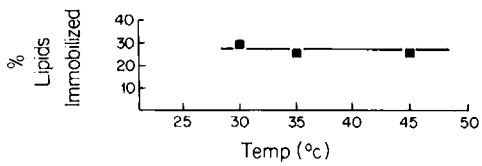

FIGURE 3

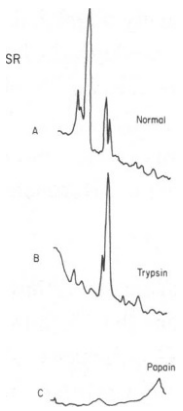

FIGURE 4

FIGURE 3 Temperature dependence of the immobilized component. The intensity of both the standard and membrane suspensions were measured as a function of temperature. The samples were allowed to equilibrate for 10 min before measuring.

FIGURE 4 Scan of SDS polyacrylamide gel electrophoresis on 10% gel of sarcoplasmic reticulum membranes stained with Coomassie blue. Track *A* is a normal preparation, track *B* has been treated with trypsin for 5 min as described in the text, and track *C* has been treated with papain for 30 min as described in the text.

Table I, no immobilization is observed. Furthermore, recombining the sarcoplasmic reticulum with egg phosphatidylcholine, as described in Methods, also produces a membrane with no detectable immobilization.

Since protein is presumed to be the source of the immobilized phospholipid detected in these experiments, a strong perturbation of protein structure might disrupt the immobilization either partially or completely. To test this suggestion, sarcoplasmic reticulum was subjected to extensive proteolysis by papain. Proteolysis of the membrane was performed in a 10 mM histidine, 1 mM cysteine, 100 mM NaCl buffer (pH 7) at 23°C. A papain/membrane protein weight ratio of 1:20 was used. Proteolysis was terminated with iodoacetamide. The extent of proteolysis was monitored with SDS polyacrylamide gel electrophoresis on 10% gels at 15, 30, 60, and 90 min. After 30 min, no further degradation took place. A gel at 30 min is shown in Fig. 4. The ^{30}P NMR spectra showed no difference in shape from Fig. 1 *C*.

With virtually complete degradation of the Ca^{++} Mg^{++} ATPase by papain, one could expect some change in the immobilization of phospholipid in the membrane if it was due to protein. Table I shows the loss of immobilization observed after proteolysis. This result supports the above hypothesis that the immobilization of phospholipid observed is due to the protein in the native membrane. (The ^{31}P NMR spectra of these membranes still exhibited the characteristic bilayer shape.)

Further characterization of the immobilized component was obtained by measuring the ^{31}P NMR spectra in the absence of NaCl of membranes that had been treated briefly with trypsin. The membranes were incubated in 10 mM histidine, pH 7, with trypsin at a 1:400 weight ratio with the membrane protein at room temperature (23°C). The reaction was terminated with a 2:1 weight ratio of soybean trypsin inhibitor to trypsin. The membranes were then washed by centrifugation in the same buffer and 10% SDS polyacrylamide gels run. A representative gel appears in Fig. 4. In contrast to the papain results, where all proteins were extensively degraded, only partial degradation of the Ca^{++} Mg^{++} ATPase has occurred and little or no degradation of the peripheral calcium binding proteins is noted. A new band arising from the Ca^{++} Mg^{++} ATPase at 56,000 overlaps the calcequestrin band. These proteolysis results are in close agreement with previously published work (30, 31). ^{31}P NMR measurements of the immobilization of these membranes appear in Table I. The ^{31}P NMR spectra show no difference in shape from Fig. 1 *C*.

^{31}P T_1

Further information about the phospholipids in the sarcoplasmic reticulum membrane can be obtained from the ^{31}P spin-lattice relaxation time, T_1, of the phospholipids in the membrane. This provides a measure of the motional rate of the phospholipid headgroup (26) and may be sensitive to lipid-protein interactions if fast exchange is taking place between the lipid-protein interface and bulk lipid (18). It should be noted that the following experiments measure the behavior only of the phospholipids that contribute to the ^{31}P resonance observed. The T_1 data are difficult to obtain because of the insensitivity of the experiment and the limitations on the amount of sample that can be introduced into the instrument. These data are obtained over a 12-h period, whereas the intensity data can be obtained in <1 h for each sample. A summary of T_1 data taken with three preparations, each from a different animal, is given in Table II. The average is 1.3 ± 0.1 s. This result is similar to that obtained for unsonicated egg

TABLE II
^{31}P NMR T_1 MEASUREMENTS

Animal	T_1*	
	Membrane	Extract
	(s)	
2	1.1	0.8
3	1.4	—
4	—	0.6
5	1.3	—
Egg phosphatidylcholine	1.3	

*±20%

phosphatidylcholine, 1.3 s, under the same conditions. This is also similar to T_1 values obtained for egg phosphatidylcholine at lower field strengths (26). ^{31}P T_1 was measured for the unsonicated chloroform-methanol extract of the sarcoplasmic reticulum and found to be somewhat shorter than for the intact membrane, but similar to the value found for the extract of the vesicular stomatitus viral membranes (18).

Phospholipid Effects on Activity

It was of interest to see whether changes in the composition of the lipids can affect enzymatic activity of the ATPase. Therefore, substitution into the immediate environment of the protein of three different classes of exogenous phospholipids was attempted. Since the major phospholipid in the sarcoplasmic reticulum membrane is phosphatidylcholine, the activities obtained with phosphatidylserine and phosphatidylethanolamine are expressed relative to the activity obtained when egg phosphatidylcholine was substituted in the same experiment using the same procedure. As will be seen, substitution into the recombinant membrane of some of the exogenous phospholipids is obtained.

The phospholipid composition of the sarcoplasmic reticulum membrane was measured as described in Methods. The composition found was: 65% phosphatidylcholine, 19% phosphatidylethanolamine, and 12% (phosphatidylserine plus phosphatidylinositol). These results agree with those obtained previously (9, 32, 33). The protein composition was verified by SDS polyacrylamide gel electrophoresis on 10% gels. A typical Coomassie blue-stained gel appears in Fig. 4. The major band has been shown to be the Ca^{++} Mg^{++}-dependent ATPase of the sarcoplasmic reticulum (34).

Phosphatidylcholine-containing Recombinants

The first exogenous phospholipid to be added was egg phosphatidylcholine. Exogenous egg phosphosphatidylcholine was added at approximately a 1:0.8 mole ratio with endogenous phospholipids. When the recombinant was analyzed for phospholipid composition, nearly 100% of the phospholipids were phosphatidylcholine, in agreement with the previous results when this recombination technique was used (9). The phospholipid/protein mole ratio in the recombinants was also determined, with 119,000 for the molecular weight of the Ca^{++} ATPase (35). This approach is reasonable, since the ATPase is purified by this procedure and no other protein appears to contribute significantly to the Lowry determination (gel

electrophoresis of the recombinants stained with Coomassie blue show predominantly one band).

The phospholipid/protein ratio shows that the membrane has been partially delipidated with respect to the original sarcoplasmic reticulum. The amount of phospholipid left with the protein varied from ~32–38 phospholipid molecules/protein molecule in these recombination experiments. No correlation was noted between the total phospholipid content and the activity of the preparation. This is not surprising because, according to previous interpretations of delipidation experiments, ~30 lipids/protein are required for maximal activity (7) and most of the present experiments produced recombinants in excess of that phospholipid requirement.

Phosphatidylserine-containing Recombinants

After confirming that phosphatidylcholine could be substituted into these recombinants, phosphatidylserine was tested for its ability to incorporate into the recombinant membrane. Exogenous phosphatidylserine was mixed with phosphatidylcholine at several mole ratios in deoxycholate and incubated with the protein as described earlier. The total amount of phospholipid added, however, was held constant at a 1:0.8 exogenous/endogenous lipid mole ratio. The recombinants demonstrated that substitution of phosphatidylserine into the recombinant was indeed possible. Increasing concentrations of exogenous phosphatidylserine led to increasing phosphatidylserine content in the recombinant membrane, as is seen in Table III. These data represent an average of five independent experiments on material from several animals for each initial mole ratio listed. The uncertainty listed is the standard deviation of the mean. Knowing the phospholipid composition of the original membranes and the mole ratio of the exogenous phospholipids, one may calculate the composition of the recombinant assuming ideal mixing of exogenous phosphatidylserine and phosphatidylcholine with endogenous membrane phospholipid. The experimentally observed mole ratios of these two phospholipids in the recombinants are predicted well by assuming that random mixing of the exogenous and endogenous phospholipids is allowed by the deoxycholate. Table III shows these calculations. This suggests that there is no preferential binding by the integral membrane protein of either of these two phospholipids with respect to each other.

The activity of the enzyme was assayed in these recombinants containing several different mole ratios of phosphatidylcholine and phosphatidylserine. A representative experiment is shown in Table IV. The activity value for the recombinant formed with 100% phosphatidyl-

TABLE III

Ca^{++} Mg^{++} ATPase RECOMBINANTS WITH PHOSPHATIDYLSERINE-PHOSPHATIDYLCHOLINE: PHOSPHATIDYLCHOLINE COMPOSITION

Exogenous phospholipid composition*	Composition in isolated recombinants*	Calculated composition*
	(%)	
100	100	84
50	64 ± 4	57
25	41 ± 1	43
0	29 ± 4	29

*Mole percentage of phosphatidylcholine.

TABLE IV

Ca^{++} STIMULATED ACTIVITY IN PHOSPHATIDYLCHOLIN-PHOSPHATIDYLSERINE RECOMBINANTS: REPRESENTATIVE EXPERIMENT

Exogenous phospholipid composition*	Activity	Activity
	(μmol ATP/mg protein/min)	*(%)*
100	5.3	100
50	3.4	64
0	2.5	47

*Mole percentage of phosphatidylcholine.

choline is similar to that reported previously for the partially delipidated sarcoplasmic reticulum membrane (9). Activity decreases as the phosphatidylserine content of the recombinant increases. A summary of all the data of this type is presented in Fig. 5. Since the predominant phospholipid components of these recombinants are phosphatidylcholine and phosphatidylserine, the observed phospholipid composition of the recombinant is presented as mole percentage of phosphatidylserine (as determined from thin-layer chromatography). Activity is presented in relative terms to normalize for differences in activity among different sarcoplasmic reticulum preparations. In each experiment a recombinant with 100 mol% (mol/100 mol) exogenous egg phosphatidylcholine was formed. The activity of this recombinant constituted the 100% activity value for that particular experiment. The activities of all other recombinants in that experiment containing some phosphatidylserine were then expressed as a percentage of the activity measured for the recombinant containing only phosphatidylcholine.

Each of the points graphed in Fig. 5 represents the average of three or four independent experiments, using material from three different animals. The line drawn through the points has no theoretical basis. However, the inhibition of Ca^{++} Mg^{++}-dependent ATPase activity by phosphatidylserine appears from these data to be best described as a linear function of the mole percentage of phosphatidylserine composition in the recombinant.

The highest composition of phosphatidylserine obtained in these experiments was about 83

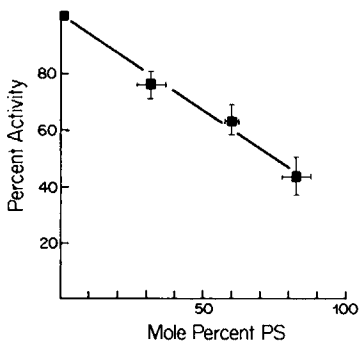

FIGURE 5 Relative Ca^{++}-stimulated ATPase activity of recombined membranes containing the Ca^{++} Mg^{++} ATPase and various compositions of phosphatidylserine, as a function of mole percentage of phosphatidylserine in the isolated recombinants. Relative activities were obtained as described in the text. Each point represents the average of three or four independent experiments.

mole percent. An attempt was made to increase the observed phosphatidylserine content by starting with twice as much exogenous phospholipid at a composition of 100 mole percent phosphatidylserine. This experiment produced a higher phospholipid/protein ratio, but no significant increase in phosphatidylserine content and no significant decrease in activity.

Phosphatidylethanolamine Incorporation

Experiments analogous to those performed with phosphatidylserine were attempted with soybean phosphatidylethanolamine to see whether the latter incorporated into the recombinants and to measure any effects on ATPase activity. As with phosphatidylserine, phosphatidylethanolamine was mixed with egg phosphatidylcholine at mole percentages of 100, 50, 25, and 0 of the latter. After isolation of the recombinants, the phospholipid composition was determined as before by thin-layer chromatography. No ninhydrin-positive spots appeared, however, in contrast to the results with phosphatidylserine recombinants and with the intact sarcoplasmic reticulum. Acid charring revealed only one dominant spot, in the position of phosphatidylcholine. These recombinants did exhibit activity, although somewhat reduced from that of the control, which had been prepared with 100 mole percent exogenous phosphatidylcholine. The reduction in activity did not exhibit any apparent relationship to the phospholipid-protein mole ratio, which was similar to that for the other recombinants reported here. There was also no apparent relationship of the activity of these recombinants to the initial composition of the exogenous phospholipids. The average activity was 84% of the 100% phosphatidylcholine recombinant formed in the same experiment.

Sufficient unsaturation exists in the hydrocarbon chains of soy phosphatidylethanolamine, so that in pure form it will exist in hexagonal (H_{II}) phase as well as a lamellar phase (36). Such a tendency may prohibit this phospholipid from incorporating into the recombinant using this procedure. When phosphatidylethanolamine is made by transphosphatidylation of egg phosphatidylcholine, it is less unsaturated in its hydrocarbon chains and is much more stable in the lamellar phase (Albert and Yeagle, unpublished results). Therefore, the recombination experiment was repeated with this less unsaturated phosphatidylethanolamine. In these experiments phosphatidylethanolamine was incorporated, as shown in Table V. But no reduction in activity was noted when compared with a control recombined with egg phosphatidylcholine alone.

TABLE V

Ca^{++} Mg^{++} ATPase RECOMBINANTS WITH PHOSPHATIDYLETHANOLAMINE-PHOSPHATIDYLCHOLINE: PHOSPHATIDYLCHOLINE COMPOSITION

Exogenous phospholipid composition*	Composition in isolated recombinants‡	Calculated composition*
100	97 ± 2	84
50	53 ± 5	57
25	39 ± 4	43
0	27 ± 4	29

*Mole percentage of phosphatidylcholine.

‡Average of three independent experiments.

DISCUSSION

The physical measurements presented here strongly suggest that there are at least two motionally distinct environments for phospholipids in the sarcoplasmic reticulum membrane. The immobilized environment is characterized by a resonance so broad that it does not significantly contribute to the intensity of the ^{31}P NMR resonance observed, whereas the rest of the phospholipid exhibits a resonance similar to dispersions of pure phospholipids.

Such a broadening of a ^{31}P NMR resonance can be caused by at least two mechanisms. In one mechanism the rate of motion of the phospholipid headgroup could be sufficiently decreased in the immobilized environment, so that dipolar contributions to the resonance line width broaden the resonance beyond detection. Alternatively, the motional freedom of the phospholipid headgroup could be decreased, which would lead to a decreased effectiveness of motional averaging of the chemical shift tensor of the phospholipid phosphate. Such an effect would increase the observed chemical shift anisotropy and thus the width of the resonance from the 45 ppm observed in the liquid crystal state towards the ~200 ppm observed in the dehydrated powder (16). Thus, these data may arise from either an immobilization due to a decreased rate of motion, to decreased freedom of motion, or to a combination of both. At this time, experimental evidence is not available to distinguish among these possibilities.

One can, however, conclude that something in the membrane is creating a special environment for some of the membrane phospholipids. Data from electron spin resonance (ESR) spin-label measurements also detect a perturbation of part of the membrane lipids (13, 14). A similar suggestion arose from an early ^{13}C NMR study (37), in which 25 ± 15% of the lipids did not contribute to the observed ^{13}C NMR resonance intensity. The most likely candidate is the protein component of the sarcoplasmic reticulum, a suggestion supported by the data obtained here from papain-treated membranes. Papain removed the immobilization of the phospholipids and concurrently completely digested the membrane proteins.

The assignment of this immobilized component to phospholipid interactions with the Ca^{++} Mg^{++} ATPase, although enticing, is probably not correct. The salt effect more clearly implicates a peripheral membrane protein than an integral membrane protein. In the proteolysis experiments, only when the peripheral membrane proteins were completely digested did the immobilization noted vanish. Finally, recent work on recombinants of purified Ca^{++} Mg^{++} ATPase with phosphatidylcholine did not detect an immobilized component (38), which once again suggests that in the intact membrane the immobilization is caused by the peripheral membrane proteins. Recent work with cytochrome *c* binding to the surface of negatively charged phospholipid vesicles has shown that peripheral membrane proteins can cause phospholipid headgroup immobilization (Yeagle, manuscript submitted for publication). The ability of KCl plus sucrose to eliminate the immobilization suggests a possible role of local salt concentrations for regulation of phospholipid-protein interactions that may, in turn, regulate enzymatic activity. The recombination experiment urges caution, in that the recombinant does not have the same structure as the original membrane with respect to lipid-protein interactions.

^{31}P NMR measurements measure most directly phospholipid headgroup behavior, as did the ^{13}C NMR study referred to above (37). Hydrocarbon chain behavior need not be the same. A phospholipid headgroup can be immobilized by the protein on the NMR time scale,

whereas the hydrocarbon chains move on and off the protein on the same time scale. On the much faster time scale of ESR, however, these same hydrocarbon chains may be immobilized by the protein.

It should be emphasized that these ^{31}P NMR measurements are made on intact sarcoplasmic reticulum, without the addition of probes, chemical modifications, or recombination procedures. This reduces the chance that these observations are artifacts. Data have been obtained from other membrane systems that further reduce the likelihood that these results represent an artifact. Glycophorin from the human erythrocyte membrane has been recombined with phospholipids at several phospholipid/protein mole ratios. A portion of the phospholipid was found immobilized by the protein in each case (19, 20). Although the percentage of the total phospholipid affected by the protein varied from experiment to experiment in accord with the phospholipid/protein ratio, the number of phospholipids immobilized per protein was constant at ~29 (20).

Only small physical effects on nonimmobilized phospholipids are detected. The ^{31}P T_1 data presented here appear to rule out strong motional affects on the T_1 time scale on the nonimmobilized phospholipid. Both the nonimmobilized membrane phospholipids and the lipid extracts have similar ^{31}P T_1 values. This T_1 can be related to the rate of rotation of the phospholipid headgroups by a previously described motional model (26). Thus, the headgroup rotational rate is not strongly affected by the presence of the protein. These ^{31}P T_1 data are distinctly different from the data obtained with vesicular stomatitus virus, where the T_1 was strongly perturbed by the membrane proteins and probably represented a rapid exchange between an immobilized and a nonimmobilized environment (18). The shape of the ^{31}P NMR spectra of the sarcoplasmic reticulum also are little different from pure phospholipid dispersions. Calorimetry data show reduction in the enthalpy of the gel-to-liquid crystalline transition of recombinants with the Ca^{++} Mg^{++} ATPase when saturated phosphatidylcholines are used, but little effect on the transition temperature. The reduction is proportional to the protein content, which may indicate strong effects on phospholipids, next to proteins, but little effects on the other lipids (15). ESR spin-label spectra likewise show evidence of both strongly perturbed lipid and nonimmobilized lipid, which is little different from pure phospholipid dispersions (13, 14). Thus, there appears to be a clear distinction between the two phospholipid environments in the sarcoplasmic reticulum. Furthermore, exchange between these environments appears to be slow on the NMR time scale. The ^{31}P NMR line shape of the nonimmobilized phospholipid is not strongly affected by the immobilized phospholipid, which implies that exchange of the headgroups between the two environments is slow (i.e., $<10^3\ s^{-1}$).

The recombinant data reported here reveal no apparent preference of the enzyme for certain phospholipids as neighbors of the protein if all the phospholipids prefer the lamellar phase. This conclusion is based on the apparent random mixing of the phospholipids during recombination. It is interesting, however, that the phospholipid preferring hexagonal phase is apparently excluded from the recombinant. If this is a basis for discrimination by the protein, it would suggest that the Ca^{++} Mg^{++} ATPase requires a phospholipid bilayer for stabilization of the proper structure for enzymatic activity, and that it may be capable of recognizing phospholipids based on their phase behavior.

These data are obtained from recombinants with low phospholipid/protein ratios, which

may introduce some unwanted protein-protein interactions. These recombinants do allow an analysis of phospholipid effects at the lipid-protein interface, however, because, most likely, nearly all the phospholipids in the recombinant are next to protein, whereas in a recombinant with a high phospholipid/protein ratio, one cannot be certain which phospholipids are immediately adjacent to the protein.

Another consideration important to this discussion is the observation that a detergent, dodecyl octaethleneglycol monoether, can activate the enzyme without any phospholipid (11). Therefore, the phospholipid requirement is called into question. The phase structure of the detergent is not well understood, however, so that which phospholipid it most resembles in phase structure is not clear. It may well be that it produces an environment for the enzyme similar to that offered by a phosphatidylcholine bilayer, which stabilized the enzyme in an active conformation.

Although these data do not provide any evidence for specific phospholipid-protein interactions based on phospholipid headgroup structure, the data with phosphatidylserine show the ability of membrane phospholipids to modulate membrane-bound enzymatic activity. The inhibition of the enzyme by phosphatidylserine may be localized in its negative charge, although no generalizations about negatively-charged lipids can be made at this time. The negative charge provides a binding site for Ca^{++} that may deplete the Ca^{++} available for the enzymes. Phosphatidylserine also may bind to lysine or arginine residues on the protein in such a way as to inhibit the enzyme. In this regard, it is interesting to observe that the data presented here do not predict complete inhibition of the enzyme by 100 mole percent phosphatidylserine. This may be an indication that the phosphatidylserine/enzyme interaction induces a lower activity conformation of the enzyme. Although such a suggestion seems intriguing in a regulatory sense, few data are available yet to support this speculation. (Inhibition by phosphatidylserine in recombinants of the Ca^{++} Mg^{++} ATPase has been reported previously [39].)

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