PERTURBATIONS OF PHOSPHOLIPID HEAD GROUPS BY MEMBRANE PROTEINS IN BIOLOGICAL MEMBRANES AND RECOMBINANTS

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ABSTRACT P-31 nuclear magnetic resonance (NMR) spin-lattice relaxation times (T_1) have been used to probe the behavior of phospholipid head groups in the presence of membrane proteins. Measurements have been made on rabbit muscle sarcoplasmic reticulum and recombinants of the Ca^{2+} Mg^{2+} ATPase, rod outer segment disk membranes and recombinants of rhodopsin, and human erythrocyte ghosts and recombinants of human erythrocyte glycophorin. Recombined membranes with lipid/protein ratios greater than or equal to that found in biological membranes showed T_1 behavior similar to the biological membranes and pure phosphatidylcholine. However, recombined membranes with a low lipid/protein ratio exhibited a T_1 that was dramatically shorter than any of the other systems. Analysis of the relaxation mechanism and the factors contributing to it implicate a phospholipid head group conformation change at high protein content. It is suggested that this is due to trapping of phospholipid between proteins and is not the same phenomenon as motional restriction at the lipid-protein interface at higher lipid contents.

INTRODUCTION

Phospholipid head group behavior in membranes has been the subject of considerable interest. Recently it has been possible to describe a model for the head group conformation of phosphatidylethanolamine, sphingomyelin, and phosphatidylcholine, which is consistent with all the available data (1, 2). The average conformation appears to place the phosphocholine moiety approximately parallel to the bilayer surface (1, 2). This conformation applies to pure phospholipid vesicles and to mixtures of phospholipids in vesicles. The effect of protein, however, on phospholipid head group conformation has not been adequately probed.

P-31 nuclear magnetic resonance (NMR) has proven to be a valuable nonperturbing means to probe the behavior of the head groups of phospholipids (1, 2, 3). Very little use has been made of the relaxation properties of the phosphorus nucleus to study phospholipid head group behavior. Studies have been made on egg phosphatidylcholine (4, 5), sphingomyelin (5), phosphatidylethanolamine (5), and dimyristoylphosphatidylcholine (6). Studies have also been made on protein-containing membranes: vesicular stomatitus virus membrane (7) and recombinants of cytochrome oxidase (6). Recently T_1 values have been reported for human erythrocyte ghosts and a crude sarcoplasmic reticulum preparation at 81 MHz (8).

Here we report a detailed study of P-31 T_1 for phospholipids in pure lipid dispersions, in three different biological membranes, and in recombined membranes containing the major proteins from each of those membranes.

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MATERIALS AND METHODS

Egg phosphatidylcholine and 1-palmitoyl-2-oleoyl phosphatidylcholine were purchased from Avanti Polar Lipids (Birmingham, AL). 3,5 diiodosalicyclic acid was obtained from Eastman Laboratory and Specialty Chemicals, Eastman Kodak Co. (Rochester, NY). Octylglucoside and deoxycholate were obtained from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, CA). Phosphate was determined by the method of Bartlett (9), and protein by the method of Lowry et al. (10).

Preparation of Human Erythrocyte Ghosts

Freshly out-of-date human red cells were obtained from the local Red Cross Blood Bank. Fresh cells were obtained from donors after an overnight fast and were drawn into 1% EDTA solution in normal saline. White or slightly pink erythrocyte ghosts were obtained by established procedures (11).

Preparation of Glycophorin and Glycophorin-Phosphatidylcholine Recombinants

Lithium diiodosalicylate (LIS) was made from 3,5 diiodosalicylic acid that was recrystallized from methanol before use. The conversion to LIS was made by the addition of LiOH in water. The LIS was recrystallized from water. Glycophorin was isolated from the human erythrocyte ghosts by the LIS extraction procedure of Marchesi and Andrews (12). The protein from each preparation was subjected to SDS polyacrylamide gel electrophoresis on 12% gels for analysis. Recombined membranes of purified glycophorin and phosphatidylcholine were prepared as described previously (13).

Preparation of Phosphatidylcholine Liposomes

Egg phosphatidylcholine or 1-palmitoyl-2-oleoyl phosphatidylcholine was dissolved in chloroform. This solution was dried under a stream of nitrogen gas and then under vacuum to a film. The film was hydrated with 10 mM histidine, 100 mM NaCl, 1 mM EDTA, pH 7, and vortexed to form a dispersion.

Preparation of Bovine Rod Outer Segment Disks

Bovine retinas were obtained from American Stores (Lincoln, NE). Rod outer segment disks were prepared as described by Smith et al. (14). All operations, including NMR measurements, were carried out in the dark or under dim red light. To reduce oxidation, 0.1 mM EDTA was added to the buffer (15). Samples were flushed with argon when storage at 4° C was required. The ratio of absorbance at 280 nm to that at 500 nm was typically 2.2.

Preparation of Rhodopsin Recombinants

Rhodopsin was purified on a Concanavalin A (Con A) affinity column after solubilizing the disks in octylglucoside. It was eluted from the column in the dark with α-methylmannoside, according to previously published procedures (16, 17). For reconstitution, egg phosphatidylcholine vesicles were first formed. Dispersions of egg phosphatidylcholine, formed as described earlier, were sonicated in a Branson W350 probe sonifier (Branson Sonic Power Co., Danbury, CT) until nearly clear. These were then centrifuged at 190,000 *g* and the clear supernatent harvested. Rhodopsin solubilized in octylglucoside was added to the vesicles, and after a half-hour incubation, the preparation was dialyzed to remove the detergent. Then the recombined membrane was applied to a 0–50% sucrose gradient and a very narrow band was observed and harvested. The recombined membrane was washed free of sucrose by centrifugation and resuspended in 100 mM NaCl.

Preparation of Rabbit Muscle Sarcoplasmic Reticulum

Sarcoplasmic reticulum was isolated from the white hind leg muscles of New Zealand white rabbits according to the procedure of Eletr and Inesi (18). This crude preparation was then fractionated on a discontinuous sucrose gradient according to previously published procedures (19). Fractions corresponding to light sarcoplasmic reticulum and heavy sarcoplasmic reticulum were harvested for measurements. ATPase activity was assayed in the presence and absence of 0.1 mM Ca^{2+} at 37°C (20). Assay medium included 125 mM KH_2PO_4, pH 7.0, 5 mM Mg^{2+}. 1 mM EGTA was used to obtain the Ca^{2+}-independent ATPase activity. CA^{2+} pumping activity was measured by assaying Ca^{2+} lost from the medium in the same buffer with the Ca^{2+}-sensitive dye, arsenazo III. Typical ATPase activities were 2 μmol P_i/mg protein/min, and Ca^{2+}/ATP ratios were ~1.3.

Preparation of Ca^{2+} Mg^{2+} ATPase Recombinant

Recombinants were made as described previously (21). 30 mg each of sarcoplasmic reticulum protein, 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-HCl, pH 8.0. This material was layered on a discontinuous sucrose gradient of 15 and 50% sucrose. The gradients were centrifuged overnight in an SW 27 rotor at 130,000 *g*. The recombinant was harvested from the lower interface, diluted in 50 mM Tris-HCl, pH 7, and washed by centrifugation at 100,000 *g* in a 50 rotor.

P-31 NMR

P-31 NMR spectra were obtained at 109 MHz on a JEOL FX 270 Fourier transform spectrometer (JEOL USA, Analytical Instruments Div., Cranford, NJ) at 30°C in 10-mm tubes. Normal spectra were obtained using a 16-pulse Hahn echo sequence with phase cycling (22). T_1 values were obtained from the inversion recovery sequence. Spectra were obtained with gated broad band noise decoupling (gated on during acquisition only). Measurements at 81 MHz were obtained on a Bruker WP 200 Fourier transform NMR spectrometer (Bruker Instruments, Inc., Billerica, MA) with a normal pulse sequence. Measurements at 40 and 25 MHz were performed as described previously (4).

RESULTS

The P-31 NMR spectrum of an egg phosphatidylcholine dispersion at 109 MHz appears in Fig. 1. A powder pattern characteristic of phospholipids in hydrated bilayers is obtained. The spectral shape has been suggested to be due to partial motional averaging of the full powder pattern by rapid axial rotation of the phospholipid head group resulting in an apparent axial symmetry (23). Further motional averaging may be achieved from limited off-axis excursions of the director. As can be seen in the remainder of Fig. 1, this spectral shape is common to biological membranes, presumably due to the phospholipid bilayer found in each. Note, however, that the P-31 resonance shape of the protein-containing membranes is slightly different than the pure phospholipids. This is probably a consequence of the T_2 relaxation. We have measured the T_2 for most of these systems and found it to be shorter by a factor of 2 to 10 in the protein-containing membranes depending on the phospholipid/protein ratio (shorter for higher protein contents). For example, in the glycophorin recombinants, the T_2 varied from ~1 to 15 ms, for the highest protein content to pure phospholipids, respectively. These T_2 values are in the same range as reported recently for other systems (6).

The primary focus of this study is the spin-lattice relaxation behavior of the phosphorus in the phospholipids. Note that in all the systems reported here, all parts of the P-31 powder pattern appeared to have the same T_1 value, in agreement with other studies (6).

The first system to be investigated was pure egg phosphatidylcholine. The T_1 as a function of field strength appears in Fig. 2. The T_1 shows no noticeable dependence on field strength over more than a fourfold range of field. The T_1 values for 1-palmitoyl-2-oleoyl phosphatidylcholine at 81 and 109 MHz are the same as for egg phosphatidylcholine. These measurements were performed on unsonicated aqueous dispersions of phosphatidylcholine. Previously it was shown that the T_1 was very similar for sonicated and unsonicated preparations (5).

To assess the influence of protein on the T_1, a wide variety of systems has been studied. These systems include both native membranes (human erythrocyte membranes, rabbit muscle sarcoplasmic reticulum [both light and heavy], and bovine rod outer segment disk membranes) and recombined membranes of either egg phosphatidylcholine or 1-palmitoyl-2-oleoyl phosphatidylcholine and

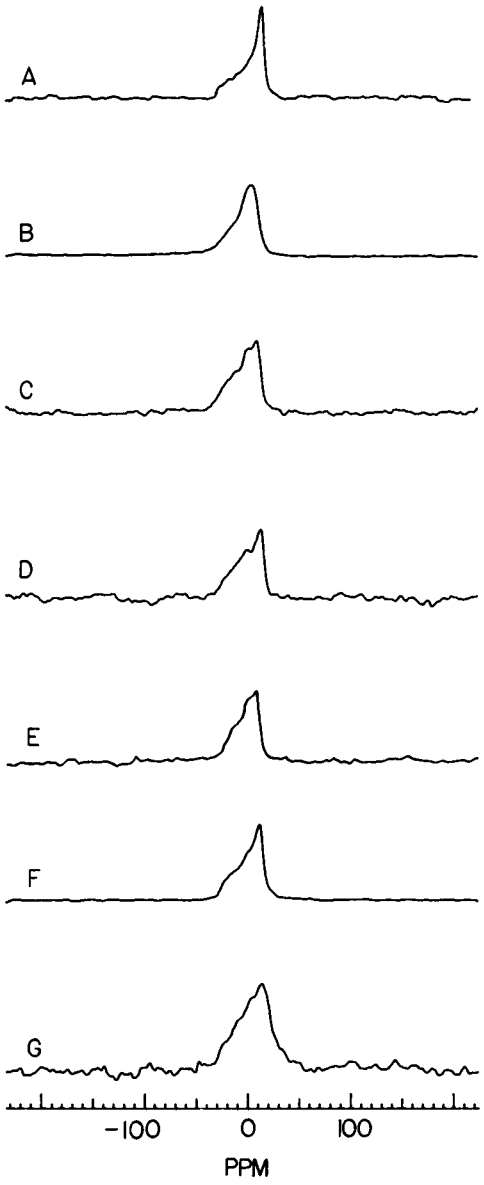

FIGURE 1 109 MHz ^{31}P NMR spectra of phospholipids and membranes at 30°C in 10-mm tubes with gated, broadband ^{1}H decoupling (gated on during acquisition only, to prevent sample heating). Line broadening of 200 Hz was used for sensitivity enhancement and only zero-order phase corrections were used. (*A*) 1-palmitoyl-2-oleoyl phosphatidylcholine; (*B*) rabbit muscle sarcoplasmic reticulum (light); (*C*) bovine rod outer segment disks; (*D*) human erythrocyte ghosts; (*E*) glycophorin recombinants with 1-palmitoyl-2-oleoyl phosphatidylcholine; (*F*) recombinants of Ca^{2+} Mg^{2+} ATPase and egg phosphatidylcholine; (*G*) recombinants of rhodopsin with egg phosphatidylcholine.

glycophorin, Ca^{2+} Mg^{2+} ATPase, and rhodopsin (of this list only erythrocytes and disk membrane have previously been studied at another field strength [8]). Thus from each biological membrane there is a corresponding recombinant of the purified major membrane protein component. All measurements on membranes containing rhodopsin were done in the dark to prevent bleaching. All T_1 measurements were made in buffers made from deionized, distilled

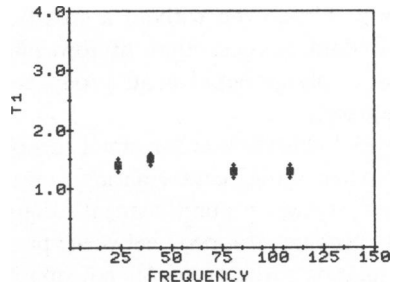

FIGURE 2 P-31 spin-lattice relaxation times for egg phosphatidylcholine in aqueous suspension as a function of frequency of measurement in megahertz.

water and containing 1 mM EDTA to exclude paramagnetic metal ions.

Fig. 3 summarizes the P-31 T_1 measurements on all the membrane systems. Little effect is noted on T_1 until a membrane with high protein content is reached. Then, suddenly, the T_1 dramatically shortens. Also plotted in Fig. 3 is a result published previously for cytochrome oxidase recombinants and pure dimyristoylphosphatidylcholine (6).

DISCUSSION

The results presented here can be discussed in a model-independent manner. The variation of T_1 with changes in correlation time (induced by temperature) has been reported for normal phospholipid bilayers at high field (24). A curve with a minimum has been observed. Thus, based on these experimental data, it is clear that a T_1 of 0.1 s cannot be reached by simply changing rates of motion. Changes in the number of protons interacting with the phosphate, or the distance of these protons from the phosphate are likely candidates for the change in T_1. Both of these require some changes in average conformation of

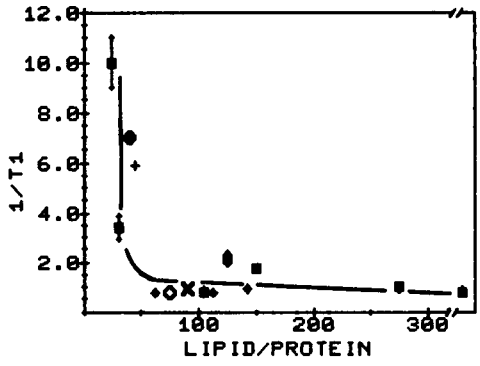

FIGURE 3 P-31 T_1 for membrane systems at 109 MHz. ■, glycophorin recombinants; ○, rod outer segment disk membranes; ●, rhodopsin recombinants; ◇, sarcoplasmic reticulum; ◆, Ca^{2+} Mg^{2+} ATPase recombinants; ×, human erythrocyte ghosts; +, cytochrome oxidase recombinants (6). The point at 340 (lipid/protein) actually represents pure phospholipids, including dimyristoylphosphatidylcholine (6), egg phosphatidylcholine, and 1-palmitoyl-2-oleoyl phosphatidylcholine. The lipid/protein ratio is a mole ratio.

the head group. Thus, even without a specific model for motion, these data suggest that at low phospholipid/protein ratios, a phospholipid head group conformation change takes place.

The next question is the mechanism of this perturbation of phospholipid head group conformation. It was suggested previously that at high protein concentrations, phospholipids would become trapped between proteins (25). Because the surface of the protein is not smooth, it would be expected that a phospholipid trapped between proteins would have forced upon it a different phospholipid head group conformation. Trapping of phospholipid by protein would be expected to exhibit a nonlinear response with respect to lipid/protein ratio. At high lipid content, no phospholipids would be trapped because the protein would be sufficiently dilute to prevent protein-protein interactions, other than their normal aggregation state in the membrane. At high protein content the amount of trapped phospholipid would increase rapidly. As seen in Fig. 3, this is the case. These results contrast with the measurements of motionally restricted phospholipid in recombinants with a lipid/protein ratio above the value where significant trapping takes place. Then the number of phospholipids motionally restricted by the protein is constant, regardless of the lipid/protein ratio (13).

Note one other interesting observation. Intact vesicular stomatitus virus exhibits a P-31 T_1 from its membrane of ~0.1 s (7). This was due to the presence of the G protein in the virus envelope (7). This short T_1 is achieved at a lipid/protein ratio of ~100:1 in the intact virus membrane (26). Thus the viral membrane protein has a powerful influence on phospholipid head group conformation at a protein content far below that required by other glycoproteins (glycophorin, rhodopsin) to have the same effect.

Recent studies with excimer formation show a distinct change in the lipid environment when the lipid/protein ratio is reduced to the values that here cause reorganization of surface structure (27). The effects noted here may also be propogated into the hydrocarbon region of the membrane.

A final question needs to be addressed. The P-31 NMR measurements reported here refer to the properties of the normal bilayer spectral component in these membranes. As we report elsewhere (28), there is a second minor, but motionally restricted phospholipid environment promoted by protein. The broad P-31 NMR resonance associated with the restricted environment is readily distinguishable from the normal bilayer resonance. Thus, the measurements reported here refer only to the properties of the less restricted phospholipids in the phospholipid bilayer of these membranes.

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