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PHOSPHOLIPID ORIENTATION IN SARCOPLASMIC MEMBRANES: SPIN-LABEL ESR AND PROTON NMR STUDIES

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SUMMARY

Three stearic acid derivatives, containing an *N*-oxyl-4',4'-dimethyloxazolidine ring attached 4, 9, or 12 carbons away from the carboxyl group, were used as spin probes in membrane vesicles obtained from sarcoplasmic reticulum.

In nuclear magnetic resonance studies, designed to establish the alignment of the probes relative to the membrane lipids, it was found that the signal originating from the choline methyl protons of the membrane phospholipids is broadened by the spin probes; this effect was found to decrease as the paramagnetic ring is farther removed from the polar end of the fatty acid probe.

The influence of the membrane structural organization on the orientation of the spin probes was studied by electron spin resonance spectroscopy. In membrane vesicles oriented by centrifugation into flat pellets, the ESR spectra indicate preferential orientation of the spin probes' long molecular axes in a direction perpendicular to the plane of the pellet.

The reported experiments establish that in sarcoplasmic reticulum membranes: (1) fatty acid spin labels are aligned parallel to and reflect the orientation of native lipids; (2) native lipids are preferentially oriented with their long axes perpendicular to the plane of the membrane.

INTRODUCTION

Vesicles of fragmented sarcoplasmic reticulum are an extremely useful system in which to study the molecular mechanisms responsible for active ionic transport. Ca^{2+} uptake, coupled to ATP hydrolysis, is the only major activity associated with these membranes. Purified sarcoplasmic reticulum vesicles consist of approximately 65 % protein and 35 % lipids. About 80 % of the latter are highly unsaturated phospholipids, the remainder being neutral lipids[1-3]. It has already been shown that sarcoplasmic reticulum membrane lipids are essential to the proper functioning of the active transport process[3-6]. If the β-ester bond of phospholipids is split by treatment of the membrane with phospholipase A, the calcium-storing ability of the vesicles is lost, but the calcium-dependent ATPase is still fully active; this enzyme is deacti-

vated, however, if the splitting products are removed with bovine serum albumin. Activity may then be restored by addition of oleic acid or lysolecithin[3,7]. The physical state of the membrane lipids was also probed at each step of these operations[8]. Spin-labelled fatty acids were added to the preparations and assumed to associate with the lipid moiety. Motion parameters, obtained from the electron spin resonance (ESR) of the stable nitroxide labels, indicate that ATPase activity requires a relatively fluid environment for the probe molecules and that this fluidity is reduced by removal of the splitting products following the phospholipase A treatment and then restored by the addition of oleic acid.

Interpretation of these results is difficult, however. No direct information is yet available concerning the equilibrium configuration of membrane lipids in sarcoplasmic reticulum. The experiments already mentioned indicate that the nitroxide probes behave differently from the manner in which they do in studies of sonicated pure phospholipid aqueous dispersions. While this may be attributed to the proteins, it may also be due to the absence of a layered arrangement of sarcoplasmic reticulum lipids or to the anomalous association of the labels with ordered lipids as has been observed in recent monolayer studies[9].

It is the purpose of this work to define the interaction of fatty acid spin probes with lipids in sarcoplasmic reticulum and to investigate whether these lipids are organized in ordered moieties with respect to the membrane surface.

MATERIALS

Sarcoplasmic reticulum was obtained from white skeletal muscle of rabbit hind legs. Soon after excision, the tissue was washed and cooled in 0.1 mM EDTA, pH 7. 150 g of trimmed muscle were then homogenized in 450 ml of Medium 1: 10 mM histidine–10 % sucrose–0.1 mM EDTA, pH 7, 15 s every 5 min, for 1 h. During this time the pH was adjusted with a few drops of 5 % NaOH when necessary.

The homogenate was centrifuged at 15000 × *g* for 20 min. The supernatant was collected and filtered through several layers of washed gauze (1-inch thick filter), to eliminate low-density lipid aggregates. The filtered suspension was then centrifuged at 40000 × *g* for 90 min and the resulting sediment was resuspended in 55 ml of Medium 2: 10 mM histidine–0.6 M KCl, pH 7.

After 40 min incubation at 2–4 °C, the suspension was centrifuged at 15000 × *g* for 20 min. After this centrifugation, the uppermost layer (approx. 10 % of the total volume) was carefully discarded, to eliminate minimal amounts of low-density lipid aggregates that may still be associated with the preparation. The remaining supernatant was then collected and recentrifuged at 40000 × *g* for 90 min. This final sediment was resuspended in 20 ml of Medium 3: 10 mM histidine–30 % sucrose. The entire procedure was carried out at 2–4 °C. The Ca^{2+}-pumping activity of sarcoplasmic reticulum vesicles stored under refrigeration remained stable for 1 week.

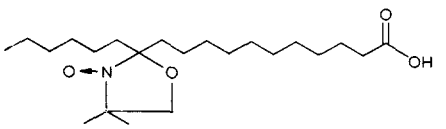
Before each spin-labelling experiment, the sarcoplasmic reticulum fragments were washed in 10 mM histidine (pH 7) and resuspended to a final concentration of approx. 100 mg protein/ml. Samples used in proton NMR studies were washed four times in ^2H_2O and resuspended to a final concentration of 30 ml of lipids/ml in a solution of 10 mM maleate and 80 mM KCl, at a p^{2}H very nearly equal to 7.

METHODS

Proton NMR spectra of sarcoplasmic reticulum preparations with and without spin-labelled fatty acid probes were measured using the 250-MHz high resolution spectrometer[10] at the National Institutes of Health Facility for Biomedical Studies, Carnegie–Mellon University. The spectrometer was operated in the frequency-swept mode and locked to the strong signal of residual ^2HHO present in all preparations. Spectra were obtained in single scans and at about 29 °C.

Electron spin resonance spectra of stable nitroxide probes in sarcoplasmic reticulum vesicle preparations were recorded on a Varian E-3 spectrometer using a rectangular tissue culture flat cell with the approximate dimensions 4.5 cm × 1 cm × 0.05 cm. One can observe paramagnetic resonance with the cell oriented with its face either parallel or perpendicular to the magnetic field in a conventional rectangular resonance cavity. Labelled sarcoplasmic reticulum preparations were centrifuged at 15000 × *g* for 10 h and the flat pellet was then transferred as carefully as possible onto the flat cell.

Three spin probes were used in this work. They are *N*-oxyl-4',4'-dimethyloxazolidine derivatives of stearic acid with the oxazolidine ring attached to the hydrocarbon chain at 4, 9 or 12 carbons, respectively, from the carboxyl group.



12-Nitroxide stearate

They are denoted in what follows by 4-, 9- or 12-nitroxide stearate. The synthesis of these probes has already been described[11] and the samples used in this work were kindly provided by Professor A. D. Keith. The probes were first dissolved in ethanol at a concentration of 10^{-3} M; equal amounts of spin-probe solutions were measured into separate test-tubes and the ethanol was then evaporated. Equal amounts of the sarcoplasmic reticulum suspension were then added to the test-tubes and agitated for 30 min before transfer to the NMR tubes or centrifugation. ESR experiments were conducted in samples with 1 spin probe for every 100 lipid molecules approximately. Samples used in NMR studies of probe interactions with membrane phospholipids contained 1 spin probe for every 6 or 24 lipid molecules approximately.

RESULTS

It has been reported earlier[12] that proton NMR spectra of concentrated (60 mg protein/ml) suspensions of sarcoplasmic reticulum vesicles display fairly well-resolved peaks that can be compared to those of sonicated dispersions of phospholipids. The main resonance lines are attributed to (CH_3), $\neg(CH_2)_n\neg$ and choline protons at −0.90, −1.26 and −3.3 ppm, respectively.

Spectra measured at 29 °C on non-labelled and labelled sarcoplasmic reticulum samples are shown in Fig. 1. The concentration of probe molecules is 1 per 6 phospholipids. Spectra were also measured at probe concentrations of 1 per 24 phospho-

lipids. The observed effects were similar, although less drastic, as the 4-nitroxide stearate label did not completely smear the choline line.

The ESR spectra of the 4-nitroxide stearate spin probe in the sarcoplasmic reticulum pellet obtained after centrifugation at 15000 × *g* for 10 h were recorded at approximately 25 °C at two orientations of the pellet with respect to the external magnetic field and are given in Fig. 2. They are characteristic of spectra due to highly

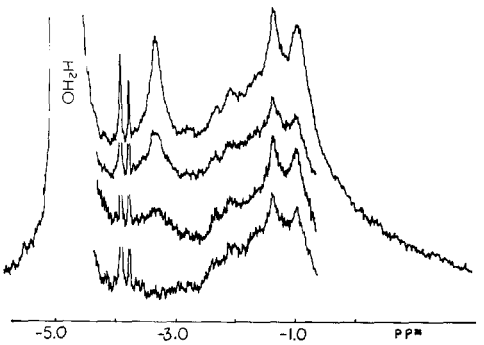


Fig. 1. High resolution NMR spectra in sarcoplasmic reticulum at 29 °C. From top to bottom: pure sarcoplasmic reticulum suspension, sarcoplasmic reticulum *plus* 12-nitroxide stearate, sarcoplasmic reticulum *plus* 9-nitroxide stearate and sarcoplasmic reticulum *plus* 4-nitroxide stearate. The concentration of spin-probe molecules is 1 per 6 phospholipids. The two sharp peaks at about −4.0 ppm are due to the buffer.

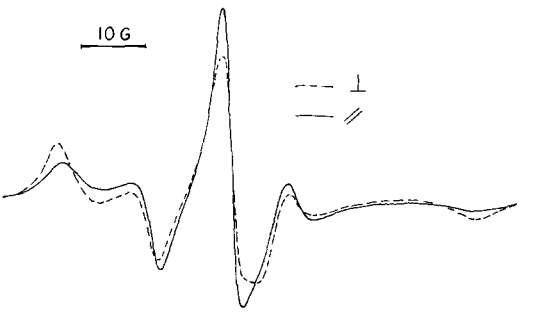


Fig. 2. ESR spectra of 4-nitroxide stearate in centrifuged pellets of sarcoplasmic reticulum vesicles oriented parallel and perpendicular to the external field.

anisotropic motion about a molecular axis (most certainly the long molecular axis) and clearly show that this axis has a definite average orientation with respect to the external magnetic field. The sense of the preferred orientation is one in which the unique hyperfine axis, *i.e.* the long molecular axis, is perpendicular to the membrane surface, *i.e.* the plane of the pellet, rather than parallel to it. It is estimated, from the relative intensities of the outer hyperfine extrema, that the spin-label orientation perpendicular to the pellet surface is more probable than that parallel to it by at least 60 %. This result is similar to that observed in the case of erythrocytes oriented by hydrodynamic shear[14].

DISCUSSION

The interpretation of all data obtained from the ESR spectra of spin-labelled probe molecules introduced *in vitro* into biological or artificial membrane preparations suffers from the same weakness, namely that the information is primarily relevant to the probe molecule itself. Implicit in these interpretations is the notion that the dynamics of the probe molecule reflect in some manner the dynamics of its immediate neighbors without appreciably perturbing the latter. In the case of labelled fatty acids, which have been extensively used to probe the lipid moiety of membranes, the underlying assumptions are that the fatty acids are dissolved for the most part in the lipid moiety, that they orient along the lipid long axis with the carboxyl group anchored to the polar region of the amphiphilic micellar or vesicular arrangement of

the lipids and that their relative freedom of motion measures the fluidity of the surrounding lipids. Recent work on the solubility of labelled fatty acids in phospholipid monolayers[9] hints that the situation may not be all that simple and that some caution must be exercised before making all these assumptions.

The results of the NMR experiments reported in this work clearly indicate that the spin-labelled fatty acids are dissolved in the lipid moiety and that they are indeed oriented preferentially along the long axis of the neighboring lipids. This is the only way in which to interpret the decreasing effect on the choline proton by the nitroxide in 4-, 9- and 12-nitroxide stearate, respectively. It should be pointed out that these data are not simply related to the positions of the nitroxide ring along the stearic acid alkyl chain, since kinetic parameters arising from transverse oscillations of the nitroxide ring, diffusion of the fatty acids within a lipid monolayer, and flip-flop motion from one monolayer to the other are expected to be different for each of the probes.

The choline proton line is of particular interest as its intensity is reversibly temperature dependent as long as the membranes are not heat denatured[12]. The sigmoidal nature of the intensity increase with temperature and the constancy of the resonance linewidth suggest a type of cooperative transition at about 40 °C[12], which correlates with a transition oberved in the temperature dependence of sarcoplasmic reticulum functional parameters, such as Ca^{2+} accumulation and ATPase activity[13]. It follows that any effect of the spin-labelled fatty acids on this choline NMR line is proof that these spin-probe molecules are indeed associated with membrane phospholipids important to the membrane's functional role. Also, since we have shown that their average orientation reflects that of neighboring lipid molecules, the results of the ESR orientation experiments may be interpreted with little ambiguity. 4-Nitroxide stearate spin probes display spectra characteristic of anisotropic rotation about their long axis[14]. Dissolved in sarcoplasmic reticulum, the average position of their long axis reflects the axial direction of lipids in the immediate neighborhood. Since sarcoplasmic reticulum vesicles have a tendency to flatten into almost biconcave discs, as revealed by negative staining electron microscopy[15,16], the centrifugation is expected to result in an ordered layering of these vesicles in planes parallel to the surface of the pellet. Thus, the orientation experiments, which clearly demonstrate that the average orientation of the spin probe's long axis is perpendicular to the plane of the pellet, indicate that the average orientation of membrane functional phospholipids is perpendicular to the surface of sarcoplasmic reticulum vesicles.

This result may be combined with others, indicating a polar environment along the entire length of the fatty acid probe[8], to derive the following picture of sarcoplasmic reticulum membrane structure. The lipids would be arranged in bilayer segments, strongly interacting with protein components which traverse the whole bilayer thickness, and thus provide a more polar environment than observed in artificial phospholipid bilayers. Evidence for protein–lipid interaction has already been found in the temperature dependence of lipid NMR lines[12] as correlated with enzymatic activity[13], and also in spin-labelling studies. The extensive nature of these interactions will be investigated in a subsequent paper.

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