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TRANSVERSE DISTRIBUTION AND MOVEMENT OF LYSOPHOSPHATIDYLCHOLINE IN SARCOPLASMIC RETICULUM MEMBRANES AS DETERMINED BY ^{13}C NMR AND LYSOPHOSPHOLIPASE

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Summary

1. The transverse distribution of 1-palmitoyl-*sn*-glycero-3-phospho-*N*-[*Me*-^{13}C]-choline in vitro incorporated in sarcoplasmic reticulum has been measured by means of ^{13}C NMR and $DyCl_3$ as an impermeable shift reagent.

2. Lysophosphatidylcholine added to the membranes equilibrates within 30 min at 20°C between outer and inner membrane leaflet so that 42% is located in the inner leaflet.

3. Lysophosphatidylcholine diffuses back from the inner leaflet to the outer upon lysophospholipase action on the outer lysophosphatidylcholine pool.

Introduction

Sarcoplasmic reticulum membranes contain enzymes capable of acylating both 1-acyl-*sn*-glycero-3-phosphocholines and 2-acyl-*sn*-glycero-3-phosphocholines [1] as well as 1-alkenyl-*sn*-glycero-3-phosphocholines [2]. This suggests that the deacylation-reacylation cycle of phosphoglycerides may be of physiological significance in sarcoplasmic reticulum. Since lysophosphatidylcholine may be a normal metabolite in sarcoplasmic reticulum, we thought it of interest to study its localization and dynamics in these membranes.

In previous papers we have shown that the transbilayer movement of lysophosphatidylcholine in artificial single bilayer vesicles and multilamellar liposomes is very slow with half-times in excess of 40 h [3—5]. Incorporation of the membrane-spanning protein glycophorin greatly facilitates the transbilayer movement of this lipid [6].

In the accompanying paper [7] we have shown that ^{13}C NMR in combination with the shift reagent $DyCl_3$ can be employed to determine the transverse distribution of phosphatidylcholine in sarcoplasmic reticulum. This technique is presently applied to determine the localization of lysophosphatidylcholine after incorporation in isolated sarcoplasmic reticulum vesicles. Subsequently, lysophosphatidylcholine movement from the inner leaflet to the outer is investigated by depletion of the outer leaflet pool by incubation of the membrane vesicles with lysophospholipase.

Experimental section

Materials

1-Palmitoyl-*sn*-glycero-3-phospho-*N*-[*Me*-^{13}C]choline (lysophosphatidyl-*N*-[*Me*-^{13}C]choline) was prepared as described by de Kruijff et al. [3]. 1-[1-^{14}C]-Palmitoyl-*sn*-glycero-3-phosphocholine was prepared according to van den Bosch et al. [8]. $^{45}CaCl_2$ was purchased from the Radiochemical Centre (Amersham, U.K.). Lysophospholipase II was purified from beef liver as described by de Jong et al. [9] Sarcoplasmic reticulum was isolated from rat hind leg muscles as described in the accompanying paper [7].

Methods

NMR. ^{13}C NMR techniques are described in the accompanying paper [7]. ^{31}P NMR measurements were performed according to de Kruijff et al. [10].

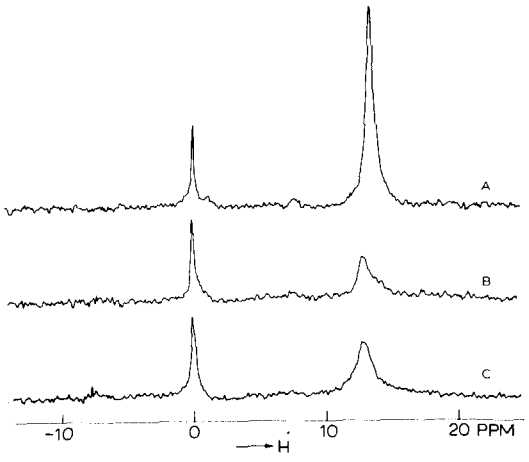
Lysophosphatidylcholine hydrolysis. Measurement of 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine deacylation by lysophospholipase treatment was carried out as described by van den Bosch et al. [11]. Complete hydrolysis was obtained by incubation of the lysophosphatidylcholine in 0.8 M NaOH for 30 min at 37°C. After acidification with HCl, the free fatty acid was extracted as described before [11].

Calcium uptake. Active Ca^{2+} uptake by sarcoplasmic reticulum was measured in a radiochemical assay using $^{45}CaCl_2$ according to Duggan and Martonosi [12].

Results

Localization of lysophosphatidyl-N-[Me-^{13}C]choline in sarcoplasmic reticulum

Micellarly dispersed lysophosphatidyl-*N*-[*Me*-^{13}C]choline containing a trace of 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine was added to sarcoplasmic reticulum. After reisolation of the membranes by centrifugation 81% of the ^{14}C label was recovered in the membrane pellet. The 90.5 MHz ^{13}C NMR spectrum of these membranes shows only one peak in addition to the peak at zero ppm due to external 1,4-dioxane (Fig. 1A). Under similar experimental conditions virtually no signal from the naturally abundant *N*-(*Me*-^{13}C) carbon atoms of endogenous phosphatidylcholine is observed. From a comparison of the peak intensity of sonicated dioleoyl phosphatidyl-*N*-[Me_3-^{13}C]choline vesicles of known concentration and the peak intensity of the sarcoplasmic reticulum membranes containing a known amount of lysophosphatidyl-*N*-[*Me*-



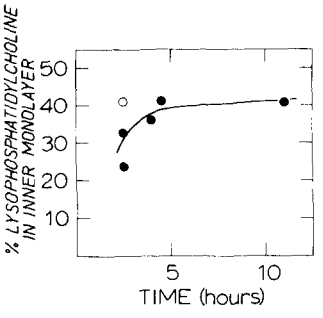


Fig. 1. 90.5 MHz ^{13}C NMR spectra of rat sarcoplasmic reticulum membranes to which 1-palmitoyl lysophosphatidyl-*N*-[*Me*-^{13}C]choline has been added. Membranes (75.6 mg protein), suspended at 0°C in 20 ml of 0.1 M KCl, 20 mM histidine, 0.02% sodium azide, pH 7.0, were incubated at 20°C for 30 min with 5 ml of 1-palmitoyl lysophosphatidyl-*N*-[*Me*-^{13}C]choline solution (10 μmol) containing a trace of lysophosphatidyl[^{14}C]choline. The membranes were immediately thereafter pelleted at 0°C by centrifugation for 1 h at 100 000 × *g*. The pellet was resuspended in 1.3 ml of the ^2H_2O analogue of the above-mentioned buffer and spectra were obtained at 30°C from 1000—3000 transients. (A) Spectrum of the membranes in the absence of Dy^{3+}, recorded 120 min after the addition of lysophosphatidylcholine to the membranes. (B) Spectrum in the presence of 3 mM $DyCl_3$ recorded 150 min after addition of lysophosphatidylcholine to the membranes. (C) As (B) except that the membranes were incubated for 7 h at 25°C in the presence of 3 mM Dy^{3+}. Chemical shifts are in ppm from external 1,4-dioxane.

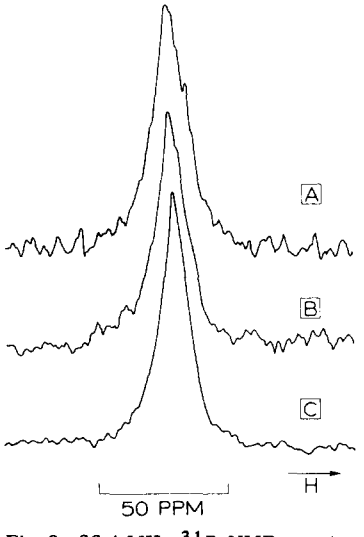
Fig. 2. Transbilayer distribution of 1-palmitoyl lysophosphatidylcholine added to rat sarcoplasmic reticulum membranes. •, experiment at 0°C in which 10 ml of a solution containing 10 μmol of 1-palmitoyl lysophosphatidyl-*N*-[*Me*-^{13}C]choline was added under stirring to sarcoplasmic reticulum (200 mg protein) suspended in 10 ml of 0.1 M KCl, 20 mM histidine, 0.02% sodium azide, 1 mM EDTA (pH 7.0). The mixture was immediately centrifuged for 1 h at 100 000 × *g* at 0°C and the pellet was resuspended in 4.0 ml of the ^2H_2O analogue of the above-mentioned buffer. The suspension was stored at 0°C. The fraction of the molecules in the inner monolayer after the indicated times was obtained from the fraction of the signal observed after the addition of 3 mM $DyCl_3$ to an aliquot of the membrane suspension. Spectra were recorded at 30°C from 1000—2000 transients. ○, experimental outlined in Fig. 1 in which the lysophosphatidylcholine was incubated with the sarcoplasmic reticulum membranes for 30 min at 20°C.

^{13}C]choline it could be calculated that 80—100% of the membrane-bound lysophosphatidyl-*N*-[*Me*-^{13}C]choline molecules are visible in the spectrum. Addition of 3 mm Dy^{3+} to the sarcoplasmic reticulum resulted in a decrease of signal intensity (Fig. 1B). The remaining signal intensity amounted to 42% of the original. Further incubation in the presence of 3 mM Dy^{3+} for 7 h had no effect on the signal intensity suggesting that the membranes did not become leaky for Dy^{3+} (Fig. 1C). For an equivalent amount of 1-palmitoyl lysophosphatidyl-*N*-[*Me*-^{13}C]choline micelles 3 mM Dy^{3+} shifted and broadened the peak so that at the original resonance position no intensity was observed any more. In the accompanying paper [7] it has been shown that 3 mM Dy^{3+} is sufficient to induce a maximum decrease in the *N*-(*Me*-^{13}C) signal intensity. We have interpreted these data as 42% of the exogenous lysophosphatidylcholine being present in the inner monolayer of the sarcoplasmic reticulum vesicles. This interpretation implies that transbilayer movement of lysophosphatidylcholine has occurred.

In order to obtain information on the rate at which the transbilayer move-

ment occurs, spectra were recorded after different incubation times at 0°C (Fig. 2). It can be seen that after 2.5 h equilibrium has not yet been reached, but after 5 h no significant increase of molecules in the inner monolayer is observed.

The integrity of the sarcoplasmic reticulum was studied by means of ^{31}P NMR. Line shape and line width of phospholipid ^{31}P NMR spectra are sensitive to the motion experienced by the ^{31}P nuclei [13]. Fig. 3A shows the 36.4 MHz spectrum of sarcoplasmic reticulum membranes. Addition of lysophosphatidylcholine up to 130 nmol/mg of protein (approx. 30% of endogenous phospholipid) induces only a slight line narrowing (Fig. 3B and C). The line width of pure lysophosphatidylcholine micelles is only about 0.12 ppm, indicating that most of the lysophosphatidylcholine added to the sarcoplasmic reticulum was bound to the membranes and that no micellization of the membranes had occurred. The observed line narrowing might be due to a slightly increased polar head group motion in agreement with observations by van Echteld (van Echtfeld, C., de Kruijff, B. and de Gier, J., unpublished results) on liposomal bilayers of dipalmitoyl phosphatidylcholine with incorporated lysophosphatidylcholine.



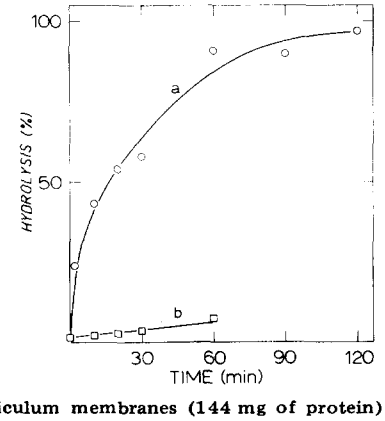


Fig. 3. 36.4 MHz ^{31}P NMR spectra of sarcoplasmic reticulum membranes (144 mg of protein) in the absence (A) and presence of (B) 10.0 μmol and (C) 18.7 μmol of lysophosphatidylcholine. The medium contained 0.1 M KCl, 20 mM histidine, 0.02% sodium azide, 1 mM EDTA (pH 7.0). To obtain the spectra 20 000 transients were accumulated at 25°C. The spectrum was recorded immediately after the addition of the lysophosphatidylcholine.

Fig. 4. Lysophospholipase action on lysophosphatidylcholine incorporated in sarcoplasmic reticulum membranes. Membranes (30.3 mg protein were incubated with 1.0 μmol of 1-[^{14}C]palmitoyl lysophosphatidylcholine (specific activity 165 dpm/nmol) for 2.5 h at 0°C in 0.1 M KCl, 20 mM histidine, 0.02% sodium azide, pH 7.0 (final volume 3.06 ml). Aliquots of 0.735 mg membrane protein were incubated with 0.4 mg of lysophospholipase II at 25°C in a final volume of 0.274 ml of the above-mentioned buffer medium. Curve a, the hydrolysis in the presence of lysophospholipase II; curve b, the blank experiment without enzyme.

TABLE I

ACTIVE Ca^{2+} UPTAKE BY SARCOPLASMIC RETICULUM AFTER INCUBATION WITH LYSOPHOSPHATIDYLCHOLINE AND LYSOPHOSPHOLIPASE

Sarcoplasmic reticulum (SR, 0.74 mg protein) was incubated for 60 min at 25°C with 24 nmol of 1-palmitoyl lysophosphatidylcholine and 0.4 mg of lysophospholipase II in 0.1 M KCl, 20 mM histidine, 0.02% sodium azide, pH 7.0 (final volume of 0.274 ml). The blank incubation did not contain lysophosphatidylcholine (LPC) and lysophospholipase. Ca^{2+} uptake was measured by addition of 0.1 μmol of $^{45}CaCl_2$ (= 1 μCi), 10 μmol of ATP, 10 μmol of Mg^{2+} (final volume of 2.0 ml). After 1 min incubation at 25°C the mixture was filtered through a millipore filter (type HA, 0.45 μm pore diameter). 100-μl samples of the filtrate were counted in 16 ml dioxane-based scintillation cocktail (10% naphthalene, 0.7% PPO, 0.03% dimethyl POPOP). $^{45}Ca^{2+}$ uptake was calculated from the decrease of the radioactivity in the filtrate.

SR (mg protein/ml)	LPC (mM)	Lysophospholipase II (mg/ml)	Incubation time (min)	Ca^{2+} uptake (μmol/mg protein)
2.7	0.09	1.46	60	0.06
2.7	—	—	60	0.06

Lysophospholipase action on lysophosphatidylcholine incorporated in sarcoplasmic reticulum

Since the transbilayer distribution of lysophosphatidylcholine in sarcoplasmic reticulum vesicles has been established, it is possible to measure the transbilayer movement of this lipid from inside to outside by lysophospholipase treatment. This is shown in Fig. 4. All lysophosphatidylcholine incubated with sarcoplasmic reticulum is hydrolyzed by lysophospholipase II within 1 h at 25°C, indicating that the inside lysophosphatidylcholine has moved to the outer surface of the vesicles, provided that the vesicles are not leaky for lysophospholipase. Active Ca^{2+} accumulation by the sarcoplasmic reticulum vesicles may be taken as an indicator of membrane integrity. Ca^{2+} uptake was measured after addition of lysophosphatidylcholine and incubation with lysophospholipase (Table I). It was found that the uptake did not significantly differ from the value for control vesicles, demonstrating that the barrier properties of the sarcoplasmic reticulum membrane are not affected by addition of the indicated amount of lysophosphatidylcholine and lysophospholipase. The value of the Ca^{2+} uptake (Table I) compares well with the uptake reported by Duggan and Martonosi [12]. Our results are also in agreement with the observations by Martonosi et al. [14] that the active Ca^{2+} uptake is only decreased at lysophosphatidylcholine concentrations above 50 μg/ml, corresponding to 2 μmol lysophosphatidylcholine/mg protein.

Discussion

In this paper we have localized lysophosphatidyl-*N*-[*Me*-^{13}C]choline bound to sarcoplasmic reticulum membranes employing a ^{13}C NMR technique described in the accompanying paper [7]. Upon addition of micellar lysophosphatidylcholine to sarcoplasmic reticulum (amounting to 132 nmol/mg protein) 81% of the added lipid was bound to the membranes. This amount of added lysophosphatidylcholine/mg membrane protein is far below the amounts necessary to impair the active Ca^{2+} uptake [14]. Of the bound lysophos-

phatidyl-*N*-[*Me*-^{13}C]choline 42% was inaccessible to Dy^{3+}. This could possibly be due to shielding of the lysophospholipid by membrane proteins. However, treatment with proteolytic enzyme (trypsin) had no effect on the accessibility to Dy^{3+}, suggesting that the protection is due to the lipid components of the vesicles. We propose that 42% of the lysophosphatidylcholine has moved across the bilayer to the inner side of the vesicles.

It is not known how lysophosphatidylcholine micelles interact with sarcoplasmic reticulum membranes. One possibility is fusion of the micelle with the membrane resulting in a simultaneous incorporation of lysophosphatidylcholine molecules in both leaflets. Alternatively, the lysophosphatidylcholine molecules are first incorporated in the outer leaflet, whereafter the molecules move to the inner side by some as yet unknown mechanism. Addition of lysophosphatidylcholine micelles to sonicated dioleoyl phosphatidylcholine vesicles results in incorporation of the lysophosphatidylcholine in only the outer monolayer of the vesicles without loss of vesicle integrity [3].

Experiments with lysophospholipase clearly showed that the lysophosphatidylcholine can move back to the outer surface of the sarcoplasmic reticulum vesicles. The half-time of this process must be less than 30 min at 25°C (Fig. 4).

It has been shown that the in vitro incorporation of radioactive fatty acids into phospholipids by rat erythrocytes occurs predominantly at the inside of the membrane [15]. If labelled lysophosphatidylcholine initially present in blood plasma was supplied to rat erythrocytes, the acylation of this lysophosphatidylcholine was mainly localized in the inner membrane leaflet, which would indicate a transposition of lysophosphatidylcholine across the membrane [16]. A similar conclusion was reached by McIntyre and Bell for the movement of another lysophospholipid, i.e. oleoyl lysophosphatidate, over the cytoplasmic membrane of *Escherichia coli* mutants [17].

The observation that also a fraction of the phosphatidylcholine pool in sarcoplasmic reticulum undergoes a rapid transbilayer movement [7] suggests that the ability of lipid molecules to move across the sarcoplasmic reticulum membrane is a general property of this membrane and is not a result of some unknown lipid-specific process.

It should be noted, however, that the conditions under which phosphatidylcholine transbilayer movement was observed [7] are different from those prevailing during lysophosphatidylcholine transverse movement as observed in this study. The phosphatidylcholine exchange experiments used to determine the transbilayer movement of phosphatidylcholine were performed under (almost) equilibrium conditions, since every phosphatidylcholine molecule removed from the sarcoplasmic reticulum is substituted by a molecule from the mitochondria [7]. In the present study a non-equilibrium condition was created either by adding lysophosphatidylcholine micelles to the sarcoplasmic reticulum or by removing lysophosphatidylcholine in the outer leaflet by lysophospholipase treatment. In both cases a membrane perturbation or a concentration gradient of lysophosphatidylcholine over the membrane was created.

The line shape of the ^{31}P NMR spectrum of sarcoplasmic reticulum (Fig. 3A) is intermediate between the line shape of membranes with a typical bilayer configuration of the phospholipids, e.g. human erythrocytes [18] and several

liposomal systems [19,20] and the line shape of liver microsomes [10,21]. The latter is indicative of significant isotropic motion experienced by the membrane phospholipids. This suggests that, although to a lesser extent than in the liver microsomes, also in the sarcoplasmic reticulum some of the phospholipids can undergo significant isotropic motion. ^{1}H NMR studies also pointed out that some 20% of the membrane phospholipids in sarcoplasmic reticulum membranes undergo isotropic motion [22]. Whether the relatively fast lysophosphatidylcholine movement across the sarcoplasmic reticulum is related to this property is as yet unknown.

Acknowledgement

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