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INSIDE-OUTSIDE DISTRIBUTION AND DIFFUSION OF PHOSPHATIDYLCHOLINE IN RAT SARCOPLASMIC RETICULUM AS DETERMINED BY ^{13}C NMR AND PHOSPHATIDYLCHOLINE EXCHANGE PROTEIN

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Summary

1. The transverse distribution of phosphatidylcholine in rat sarcoplasmic reticulum was investigated employing ^{13}C NMR in conjunction with the shift reagent $DyCl_3$.

2. Sarcoplasmic reticulum phosphatidylcholine was enriched with ^{13}C by feeding rats a diet containing [*N*-*Me*$_3$-^{13}C]choline. Up to 32% of the sarcoplasmic reticulum phosphatidyl-[*N*-*Me*$_3$-^{12}C]choline was replaced by phosphatidyl-[*N*-*Me*$_3$-^{13}C]choline.

3. Titration of ^{13}C-enriched sarcoplasmic reticulum with Dy^{3+} indicates that 40% of the phosphatidyl-[*N*-*Me*$_3$-^{13}C]choline is exposed to the external medium, whereas 60% is shielded from interaction with Dy^{3+}.

4. Incubation of ^{32}P-labelled sarcoplasmic reticulum with excess mitochondria and phosphatidylcholine exchange protein results in a fast transport of approx. 80% of [^{32}P]phosphatidylcholine to the mitochondria indicating that part of the phosphatidylcholine pool is involved in a rapid transbilayer movement.

Introduction

Since the discovery of an asymmetric transbilayer distribution of the phospholipids in red blood cell membranes [1—4], many other biological membranes have been studied with respect to their phospholipid distribution. These include other mammalian plasma membranes [5,6], subcellular membranes [7,8,41,42], bacterial membranes [9,10,11,13] and viral envelopes [14—16].

For this purpose several different probing techniques were employed, such as chemical reagents [1,3,5,8,10,13,15,16], phospholipases [2,4,6,7,9,11], exchange proteins [5,9,17], and ESR techniques [18,19]. In essence all techniques are based on the use of some, supposedly non-permeating, reagent which interacts with one side of the membrane only. However, all probes more or less perturb the native membrane structure and may give rise to artefacts. This is best illustrated by the fact that three different transverse distributions were proposed for rat liver microsomes based on different phospholipase treatments [20—22].

Phospholipase treatments may induce phospholipid transmembrane movements [11,12]. Phospholipid exchange catalyzed by specific proteins, which are thought to be much milder probes, may also be difficult to be interpreted in view of a possible, relatively fast transverse diffusion of phospholipids in the unperturbed, native membrane [23,24]. NMR techniques have been employed to determine the transbilayer distribution of phospholipids in artificial single bilayer vesicles [25—30]. Especially ^{13}C NMR is useful when ^{13}C-labelled phospholipids are employed in mixtures of several lipid species. The ^{13}C resonance of the *N*-methyl groups of phosphatidylcholine is particularly useful since it is well resolved in the spectrum due to the high internal motion of the methyl groups. This permits the study on the transbilayer distribution of phosphatidylcholine in much larger structures than the small sonicated vesicles used untill now. Recently, this technique was successfully applied to large protein-free and protein-containing unilamellar vesicles [31].

In the present study we investigate the possibility of applying ^{13}C NMR in conjunction with the shift reagent Dy^{3+} to biological membranes. Specifically an attempt is made, to determine the transverse distribution of phosphatidylcholine in fragmented sarcoplasmic reticulum, liver endoplasmic reticulum and red blood cells. Only in the case of sarcoplasmic reticulum a positive result could be obtained.

Knowing the transverse distribution of phosphatidylcholine in sarcoplasmic reticulum, specific phosphatidylcholine exchange protein can be employed to investigate the possible occurrence of transverse diffusion of this phospholipid.

Materials and Methods

Preparation of membranes. Rat livers were homogenized in 0.1 M KCl, 20 mM Tris-HCl, 1 mM EDTA (pH 7.4) using a Potter-Elvehjem tube. Rat liver microsomes were prepared from a 10% homogenate by differential centrifugation. The first centrifugation step involved a force of 10 000 × *g* for 20 min. The microsomes were precipitated from the resulting supernatant by centrifugation at 105 000 × *g* for 60 min. The pellet was resuspended in the above-mentioned buffer. Liver microsomes prepared for ^{13}C NMR studies were isolated using a 0.1 M KCl buffer, since sucrose gave an intense ^{13}C NMR spectrum overlapping the [*N*-Me_3-^{13}C]choline resonance. Sucrose should therefore be avoided in such studies.

Rat hind leg muscles were homogenized at 0—4°C for 2 min in 0.1 M KCl, 20 mM histidine (pH 7.0) using a Waring blendor. The homogenate was centri-

fuged for 20 min at 15 000 × *g*. The supernatant was centrifuged for 90 min at 59 000 × *g*. The pellet was resuspended in 0.1 M KCl buffer and again centrifuged for 20 min at 15 000 × *g*. The supernatant was centrifuged for 90 min at 59 000 × *g*. The pellet was resuspended in 0.1 M KCl buffer. This preparation is referred to as sarcoplasmic reticulum or muscle microsomes.

Trypsin-treated sarcoplasmic reticulum membranes were obtained by incubating 132 mg of membrane protein with 2 mg of trypsin for 1 h at 25°C in 6 ml of 0.1 M KCl, 20 mM histidine, 0.02% sodium azide, 1 mM EDTA (pH 7.0). To prepare disrupted sarcoplasmic reticulum the membrane preparation was sonicated three times for 30 s at 0°C.

Fresh samples of blood were obtained from ether-anaesthesized rats by cardiac puncture, using acid/citrate/dextrose as anticoagulant. The erythrocytes were isolated by centrifugation at 4°C for 15 min at 5000 × *g*. The cells were washed three times in 0.1 M KCl, 20 mM histidine, 1 mM EDTA buffer (pH 7.0).

Diet experiments. [*N*-*Me*$_3$-^{13}C]Choline and (*N*-*Me*$_3$-^{13}C)-labelled 1,2-dioleoyl-*sn*-glycero-3-phosphocholine were synthesized as described by De Kruijff et al. [32]. Rats were fed a choline-deficient diet supplemented with [*N*-*Me*$_3$-^{13}C]-choline as described by Arvidson et al. [33]. To 167 g of choline-deficient diet (ICN Pharmaceuticals Inc., Cleveland OH) 4 mmol of [*N*-*Me*$_3$-^{14}C]choline (90 atom% ^{13}C) and 50 μC$_i$ (0.96 μmol) of [*N*-*Me*$_3$-^{13}C]choline (The Radiochemical Centre, Amersham, U.K.) were added. This diet was given for 8 days to two male Wistar rats. The rats were then killed and the membranes were isolated as described above.

Analytical procedures. Lipids extracted by the Bligh and Dyer procedure were separated by two-dimensional thin-layer chromatography [34]. Lipid phosphorus was determined according to Rouser et al. [35]. Radioactivity was measured by counting the scraped spots in toluene-based scintillator/Triton X-100/H_2O (2 : 1 : 0.2, v/v/v) using a Packard Tricarb liquid scintillation spectrometer.

^{13}C NMR. 90.5 MHz ^{13}C NMR measurements were performed at 25°C on a Bruker WS-360 spectrometer under conditions as described in detail before [30—32]. Prior to the NMR experiment 10% (v/v) of the ^2H_2O analogue of the used buffer was added to the membrane preparation. Chemical shifts are reported from external dioxane. This standard is also employed for the determination of the relative peak intensity of the choline resonance. The estimated error in the determination of the peak intensity is 10%.

Exchange experiments. Beef liver phosphatidylcholine exchange protein was purified according to Kamp et al. [36]. It was a generous gift from Dr. K.W.A. Wirtz and Mr. J. Westerman. Rat liver mitochondria were isolated as described by Wirtz and Zilversmit [37]. Male Wistar rats were injected intraperitoneally with 1 mCi of carrier-free, isotonic sodium [^{32}P]orthophosphate (The Radiochemical Centre, Amersham, U.K.) per rat before removal of the hind leg muscles. Incubations of ^{32}P-labelled sarcoplasmic reticulum with unlabelled mitochondria and phosphatidylcholine exchange protein were carried out at 25°C in 0.25 M sucrose, 20 mM Tris-HCl, 0.02% sodiumazide (pH 7.2). Mitochondria were subsequently precipitated by centrifugation at 15 000 × *g* for 5 min. The supernatant was carefully pipetted off and extracted by the

Bligh and Dyer procedure. The lipid extract was chromatographed according to Broekhuyse [34]. The phosphatidylcholine and phosphatidylethanolamine spots were scraped and counted in toluene (0.5% PPO, 0.03% dimethyl POPOP)/ Triton X-100/water (2 : 1 : 0.2, v/v/v).

Ca^{2+} uptake. $^{45}Ca^{2+}$ uptake was measured as described by Duggan and Martonosi [38]. $^{45}CaCl_2$ was obtained from The Radiochemical Centre (Amersham, U.K.).

Results

Transverse distribution of phosphatidylcholine in sarcoplasmic reticulum as probed by ^{13} C NMR

The natural abundant (N-Me_3-^{13}C) resonance of phosphatidylcholine in sarcoplasmic reticulum membranes can be resolved in the 90.5 MHz ^{13}C NMR spectrum (Fig. 1A). Part of the signal intensity is lost upon addition of 3 mM Dy^{3+} (Fig. 1B). Approximately 50% of the original signal is remaining, even after 4 h incubation in the presence of Dy^{3+}. After sonication of the membranes in the presence of Dy^{3+} all signal is lost (Fig. 1C). Since the natural abundance of ^{13}C is only 1.11%, the signal intensity and signal/noise ratio are low. To improve the intensity, the amount of N(Me-^{14}C) label in sarcoplasmic reticulum phosphatidylcholine was increased by feeding rats a diet containing (90 atom% ^{13}C) [N-Me_3-^{13}C]choline. The incorporation of ^{13}C label was followed by inclusion of a trace amount of [N-Me_3-^{13}C]choline in the diet. An analysis of the phospholipids isolated from the hind leg sarcoplasmic reticulum is given in Table I. From the incorporated ^{14}C radioactivity the ^{13}C abundance in the methyl groups of the choline moiety of the phosphatidylcholine was calculated to amount to 12.5%.

Sphingomyelin was the only other phospholipid that was significantly

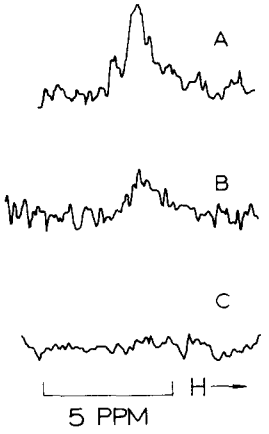

Fig. 1. Natural abundance (N-Me_3-^{13}C) resonance of sarcoplasmic reticulum membranes at 90.5 MHz. The sarcoplasmic reticulum membranes were suspended at a concentration of 80 mg protein/ml in 0.1 M KCl, 20 mM histidine, 0.02% sodiumazide, 1 mM EDTA (p^{2}H 7.0) using a Potter-Elvehjem tube. Spectra were recorded at 25°C using 6000 transients with a 1 s interpulse time. (A) Sarcoplasmic reticulum membranes in the absence of Dy^{3+}. (B) In the presence of 3 mM Dy^{3+}. The chemical shift of the (N-Me_3-^{13}C) resonance is 12 ppm upfield from external 1,4-dioxane.

TABLE I

PHOSPHOLIPID ANALYSIS OF SARCOPLASMIC RETICULUM FROM RATS FED WITH [*N*-Me_3-^{13,14}C]CHOLINE

The analysis relates to the experiment described in Materials and Methods in which two rats were fed with 4 mmol of [*N*-Me_3-^{13}C]choline and 0.96 μmol of [*N*-Me_3-^{14}C]choline. Sph, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine and PE, phosphatidylethanolamine.

Class	Composition (mol%)	^{14}C distribution (%)	^{14}C specific activity (cpm/nmol)	Choline methyl ^{13}C abundance (%)
Sph	4.2	4.2	2.0	9.0
PC	67.4	94.3	2.8	12.5
PI	4.7	—	—	—
PS	2.7	—	—	—
PE	21.0	0.9	0.08	—

labelled (Table I). The diet experiment was carried out once more employing twice the amount of [*N*-Me_3-^{13}C]choline. In this case the ^{13}C abundance of the phosphatidylcholine choline methyl groups was found to be 32%. Fig. 2A shows the large improvement of the signal/noise ratio in the ^{13}C NMR spectrum of the sarcoplasmic reticulum membranes from rats fed with [*N*-Me_3-^{13}C]-choline.

The peak intensity of the choline methyl signal/μmol of ^{13}C was compared with the intensity of the same signal from sonicated dioleoyl phosphatidyl-[*N*-Me_3-^{13}C]choline vesicles (per μmol of ^{13}C). The former amounted to 98% of the latter intensity, indicating that virtually all (*N*-Me_3-^{13}C) signals from the sarcoplasmic reticulum are observed in the spectrum.

Fig. 2 B—D illustrates the effect of increasing concentrations of $DyCl_3$ on the ^{13}C NMR spectrum of the (*N*-Me_3-^{13}C)-labelled sarcoplasmic reticulum. The observed decrease of the choline methyl ^{13}C signal is due to an increasing fraction of the nuclei interacting with the paramagnetic ion. A plot of the integrated peak intensities as a function of the Dy^{3+} concentration is shown in Fig. 3. A plateau is reached at concentrations ranging from 1 to 3 mM followed by an abrupt fall in intensity beyond 3 mM. This discontinuity coincides with the appearance of a visible precipitation of the sarcoplasmic reticulum. Below a concentration of 3 mM Dy^{3+} the sarcoplasmic reticulum suspension is relatively stable as judged from the suspension turbidity (measured at 450 nm).

The titration curve shown in Fig. 3 is interpreted by us as 40% of the phosphatidylcholine molecules being present in the outer monolayer of the sarcoplasmic reticulum vesicles. The remaining 60% is protected from interaction with Dy^{3+} by localisation in the inner monolayer. Shielding by membrane proteins on the outer surface is unlikely, since a titration curve of trypsin-treated sarcoplasmic reticulum was found to be similar to the curve shown in Fig. 3 for intact membranes.

Incubations up to 4 h in the presence of 2—3 mM Dy^{3+} did not affect the intensity of the choline signal, indicating that the permeability barrier to Dy^{3+} remains intact. This barrier was demolished by sonication of the sarcoplasmic reticulum in the presence of 2 mM Dy^{3+} as indicated by a decrease in signal

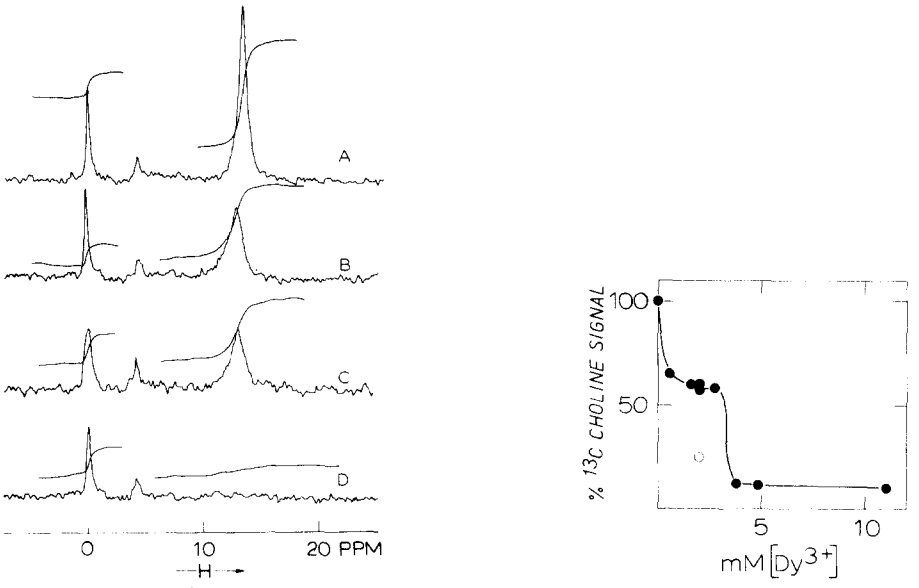

Fig. 2. 90.5 MHz ^{13}C NMR spectra of sarcoplasmic reticulum membranes, isolated from [*N*-*Me*$_3$-^{13}C]-choline-fed rats in the absence (A) and in the presence of 1.6 mM $DyCl_3$ (B), 2.7 mM (C) and 3.8 mM (D). The membranes (22.4 mg protein/ml) were suspended in 0.1 M KCl, 20 mM histidine, 0.02% sodium-azide, 1 mM EDTA (p^2H 7.0). The indicated Dy^{3+} concentrations are free Dy^{3+} concentrations obtained from the amount of added $DyCl_3$ corrected for the amount of EDTA present in the buffer. Spectra were recorded at 25°C using 1000 transients with a 1 s interpulse time. The resonance at 4 ppm originates from histidine present in the buffer. Chemical shifts are upfield from external 1,4-dioxane. Integrated peak intensities are shown superimposed on the spectra.

Fig. 3. Effect of the Dy^{3+} concentration on the intensity of the choline resonance in sarcoplasmic reticulum membranes obtained from rats which had been fed a choline-deficient diet supplemented with [*N*-*Me*$_3$-^{13}C]choline. ○, the intensity of the choline signal after two times freezing and thawing the sample. For further experimental details see legend to Fig. 2.

intensity to 10% of the initial intensity. A similar effect was observed after two freeze-thaw cycles of the sample (Fig. 3). According to our interpretation of the titration curve (Fig. 3), the abrupt decrease of choline signal intensity is caused by an increased permeability of the membrane toward Dy^{3+}. To investigate this further we have probed the effect of Dy^{3+} on the important function of sarcoplasmic reticulum, the active uptake of Ca^{2+}. Fig. 4 shows the effect of two Dy^{3+} concentrations on the amount of Ca^{2+} accumulated by the sarcoplasmic reticulum. At low concentration (0.5 mM) Dy^{3+} has not much of an effect, but at relatively high concentration (6 mM) the Ca^{2+} content is clearly decreased.

^{13}C NMR applied to rat liver microsomes and erythrocytes

In an attempt to apply the ^{13}C NMR technique to other membranes, also rat liver microsomes and erythrocytes were investigated. In the diet experiment [*N*-*Me*$_3$-^{13}C]choline was incorporated in rat liver microsomes to an even larger extent than in the sarcoplasmic reticulum. An intense (*N*-*Me*$_3$-^{13}C) resonance was observed (not shown). However, addition of Dy^{3+} to the liver microsomes resulted in complete loss of signal intensity even at low (0.7 mM) Dy^{3+} con-

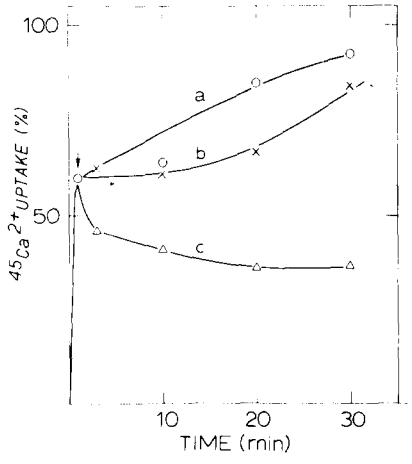

Fig. 4. Ca^{2+} uptake by sarcoplasmic reticulum (a) in the absence of Dy^{3+}; (b) with 0.5 mM Dy^{3+}, and (c) with 6 mM Dy^{3+}. The uptake is plotted as the percent of the total amount of Ca^{2+} present in the incubation mixture. Dy^{3+} was added (arrow) 1 min after a preincubation at 25° C during which Ca^{2+} was actively accumulated. The incubation mixture contained 0.37 mg/ml microsomal protein, 5 mM $MgCl_2$, 5 mM ATP, 0.05 mM $^{45}CaCl_2$, 0.1 M KCl, 20 mM histidine, 0.02% sodiumazide (pH 7.0).

centrations, accompanied by a strong increase of the suspension turbidity (measured at 450 nm). Liver microsomal proteins might cause the observed aggregation. However, pretreatment of the microsomes with proteolytic enzymes did not abolish the Dy^{3+}-induced aggregation. A ^{13}C NMR spectrum was recorded from red blood cells isolated from the rats fed with [*N*-Me_3-^{13}C]-choline. Although a considerable choline signal was observed, it was partially overlapping with natural abundant ^{13}C resonances from hemoglobin, which made it practically impossible to measure the transbilayer distribution of the choline-phospholipids.

Exchangeability of muscle microsomal phosphatidylcholine

The following experiments are concerned with the fraction of the muscle microsomal phosphatidylcholine, that can be exchanged from the outer surface of the microsomes by means of specific phosphatidylcholine exchange protein. For such experiments another membrane preparation is required, whose phosphatidylcholine can be exchanged for the muscle microsomal phosphatidylcholine. At first we employed sonicated phosphatidyl[^{14}C]choline vesicles. From the transfer of ^{14}C label to the muscle microsomes we calculated the pool size of exchangeable microsomal phosphatidylcholine, assuming that (a) only the outer monolayer of the sonicated vesicles is involved in the exchange process and that (b) no net transfer of phosphatidylcholine in either direction occurs. The results of these experiments were not very reproducible, however. Exchangeable fractions were calculated ranging from 0.4 to 1.0. In some cases values exceeding 1.0 were obtained (which is theoretically impossible), indicating that considerable errors are involved. Although this experimental system does not seem to be very suitable for determination of exchangeable pool sizes, we have employed it to investigate the permeability properties of the muscle microsomes. A correct interpretation of the calculated pool sizes requires that

the exchange protein does not have access to the inner surface of the microsomal vesicles. The 'dextran-accessible space' of the muscle microsomes was employed as an index of membrane leakiness to macromolecules. This parameter was determined after incubation of muscle microsomes with sonicated egg phosphatidylcholine vesicles and phosphatidylcholine exchange protein under conditions which were also used for determination of the exchangeable phosphatidylcholine pool of the microsomes. Table II shows that the dextran-inaccessible space is not decreased by the incubation, indicating that the permeability is not increased. The value of about 55% of the total water space in the microsomal pellet which cannot be penetrated by the dextran compared well with the data reported by Duggan and Martonosi [38]. We consider this as an indication that the exchange protein does not have an access to the inner compartment of the muscle microsomes.

Another experimental system was attempted for the determination of the exchangeable phosphatidylcholine pool size of muscle microsomes. Muscle microsomes, labelled with ^{32}P in vivo, were incubated with liver mitochondria and exchange protein. The exchangeable phosphatidylcholine fraction was determined from the amount of [^{32}P]phosphatidylcholine remaining in the microsomes after incubation. Mitochondria were present in large excess in order to prevent significant back flow of [^{32}P]phosphatidylcholine. After incubation the mitochondria were removed by differential centrifugation. In the experiment shown in Fig. 5, only 80% of mitochondrial phospholipid was in the pellet. The remaining 20% in the supernatant contaminated the sarcoplasmic reticulum membranes. The contaminating fraction appeared to be constant for all incubations of a given mitochondrial preparation. Longer centrifugation times were not employed since these would reduce the recovery of the sarcoplasmic reticulum in the supernatant. The recovery of the sarcoplasmic reticulum was determined by the amount of [^{32}P]phosphatidylethanolamine. Since the exchange protein is specific for phosphatidylcholine, [^{32}P]-phosphatidylethanolamine transfer is not stimulated [36] so that this lipid can

TABLE II

MUSCLE MICROSOMAL WATER COMPARTMENTS AFTER PHOSPHATIDYLCHOLINE EXCHANGE

Rat hind leg muscle microsomes (9.9 mg of protein, 3.1 μmol of phosphatidylcholine) were incubated with sonicated egg phosphatidylcholine vesicles (5.5 μmol of phosphatidylcholine) in the presence or absence of 37 μg of phosphatidylcholine exchange protein. Incubations were carried out at 25°C in a total volume of 1.65 ml containing 0.1 M KCl, 20 mM histidine, 0.02% sodium azide) (pH 7.0). After the incubations dextran (purum. M_r 70 000, obtained from Fluka) and 0.5 μCi of [*carboxyl*-^{14}C]dextran (M_r 70 000, specific activity 1.043 mCi/g, obtained from New England Nuclear) were added to a final concentration of 15 mg dextran/ml. The suspensions was then centrifuged for 2 h at 160 000 × *g*. Pellets and supernatants were collected and analyzed for total water content and radioactivity. The inaccessible space is obtained by subtraction of the dextran space from the total water space.

Phosphatidylcholine exchange protein	Incubation time (min)	Total water (μl/mg dry wt.)	Dextran space (μl/mg dry wt.)	Dextan-inaccessible space/total space (μl/μl)
+	10	3.6	1.7	0.52
+	30	4.0	1.7	0.57
+	60	3.7	1.6	0.55
—	—	3.7	1.7	0.55

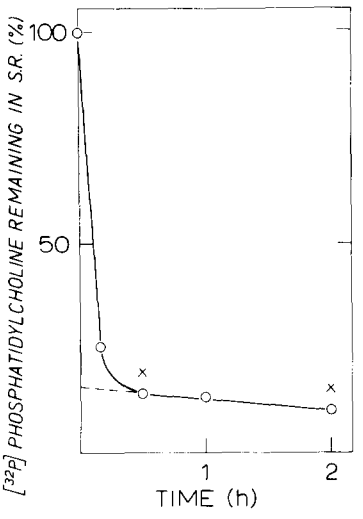

Fig. 5. Transfer of [^{32}P]phosphatidylcholine from rat sarcoplasmic reticulum membranes to rat liver mitochondria catalyzed by beef liver phosphatidylcholine exchange protein. Sarcoplasmic reticulum membranes were isolated 2 days after injection of sodium [^{32}P]orthophosphate. Sarcoplasmic reticulum membranes (439 nmol of phospholipid) were incubated with mitochondria and exchange protein (133 μg) at 25°C in 0.25 M sucrose, 20 mM Tris-HCl, 0.02% sodiumazide, pH 7.2 (final volume 3.6 ml). Either 3.0 μmol of mitochondrial phospholipid were present (X) or 7.6 μmol (○———○). Phosphatidylcholine and phosphatidylethanolamine radioactivities remaining in the supernatant after centrifugation were measured. The fractions of mitochondria and sarcoplasmic reticulum remaining in the supernatant were 0.2 and 0.88, respectively. From these data the fraction of [^{32}P]phosphatidylcholine remaining in the sarcoplasmic reticulum was calculated as indicated in the text.

be used as an internal standard. The recovery of the sarcoplasmic reticulum was found to be 80—90%. The amount of [^{32}P]phosphatidylcholine remaining in the supernatant of the incubation mixture (R_t) can be expressed as $R_t = a(1 - x)\ R_0 + b \cdot x \cdot R_0$, where R_0 is the amount of [^{32}P]phosphatidylcholine originally present in the sarcoplasmic reticulum, x the fraction of R_0 transferred to the mitochondria, a the fraction of the sarcoplasmic reticulum remaining in the supernatant and b the fraction of the mitochondria remaining in the supernatant. From this expression x can be calculated. In Fig. 5 the value of $1 - x$ is plotted as a function of the incubation time. The exchange process is biphasic: after a rapid decrease, $1 - x$ falls only very slowly. The slow phase was extrapolated to zero-time incubation. From Fig. 5 it can be seen that 85% of the sarcoplasmic reticulum [^{32}P]phosphatidylcholine is readily available for exchange.

It is possible, however, that the ^{32}P label was not yet homogeneously distributed over all phosphatidylcholine pools in the sarcoplasmic reticulum which was isolated 2 days after injection of [^{32}P]orthophosphate in the rats. Therefore the previous experiment was repeated, but this time using muscle microsomes isolated 8 days after injection of the [^{32}P]orthophosphate. This period is equal to the length of the diet experiment in which [*N*-*Me*$_3$-^{13}C]choline was administered. The results of this experiment are summarized in Table III. In this experiment 83% of [^{32}P]phosphatidylcholine was transferred to the mitochondria in the fast phase of the process.

TABLE III

EXCHANGEABLE FRACTION OF SARCOPLASMIC RETICULUM [^{32}P]PHOSPHATIDYLCHOLINE

Meaning of abbreviations and symbols: SR, sarcoplasmic reticulum; PLP, phospholipid phosphorus; *a*, fraction of SR remaining in the supernatant after centrifugation; *b*, fraction of mitochondria in the supernatant; T_0, original ratio of [^{32}P]phosphatidylcholine to [^{32}P]phosphatidylethanolamine in SR; T_e, ratio of [^{32}P]phosphatidylcholine to [^{32}P]phosphatidylethanolamine in the supernatant extrapolated from slow-phase exchange to zero incubation time; X_e, [32P]phosphatidylcholine fraction transferred to the mitochondria in the fast phase of the exchange (see text). Hind leg SR was isolated 8 days after injection of 4 mCi of sodium [^{32}P]orthophosphate in two male Wistar rats. Further experimental conditions are given under Fig. 5.

SR (μmol PLP)	Mitochondria (μmol PLP)	*a*	*b*	T_0 cpm/cpm	T_e cpm/cpm	X_e
0.4	7.7	0.8	0.08	8.2	2.1	0.83

Discussion

In this study we have presented evidence indicating that part of the endogenous phosphatidylcholine pool of rat skeletal muscle microsomes is located in the inner monolayer of the vesicles. From the natural abundance ^{13}C NMR spectra a value of approximately 50% phosphatidylcholine in the inner monolayer was calculated. When the membranes were enriched with phosphatidyl-[*N*-*Me*$_3$-^{13}C]choline by means of a diet experiment, the sensitivity of the method was greatly increased and a value of 60% of phosphatidylcholine in the inner monolayer was found. Although it seems highly unlikely, the possibility cannot be excluded that the ^{13}C label was not yet uniformly distributed over all phosphatidylcholine pools, after 8 days. The half-time of phosphatidylcholine turnover in rat sarcoplasmic reticulum is in the range of 10—15 days [39].

The success of the described method for determination of the transbilayer distribution of phosphatidylcholine is dependent on the membrane's permeability barrier for Dy^{3+}. At least part of the vesicles does not give access to their inner compartments at Dy^{3+} concentrations up to 3 mM (Fig. 3). The possibility that all phosphatidylcholine is in the inner monolayer with 40% of the vesicles being leaky for Dy^{3+} cannot be excluded, although it is not very likely. Krasnow [40] provided circumstantial evidence that trivalent gadolinium cations interact only with the outer surface of muscle microsomes and are not free in the internal space. At low Dy^{3+} concentrations (0.5 mM) the membrane structure is not seriously affected, as judged from the unimpaired Ca^{2+} pumping activity (Fig. 4).

It has been reported that as much as 87% of rabbit sarcoplasmic reticulum phosphatidylethanolamine is readily available for reaction with a non-penetrating, water-soluble complex of fluorescamine with cycloheptaamylose [41]. It was suggested that most of the phosphatidylethanolamine was located in the outer leaflet of the membrane bilayer [41]. Recently it was reported that treatment of right-side out and inside-out rabbit sarcoplasmic reticulum vesicles with phospholipase A_2, phospholipase C or 2,4,6-trinitrobenzenesulfonate gave results which are consistent with phosphatidylethanolamine being preferen-

tially located in the outer leaflet of the right-side out vesicles [42]. These and our data suggest that phospholipid asymmetry is present in the membranes, with phosphatidylethanolamine enriched in the outer monolayer and phosphatidylcholine (slightly) enriched in the inner monolayer. This asymmetry is opposite to that found in erythrocyte membranes [2]. This may be related to the fact that sarcoplasmic reticulum is an endoplasmic membrane and its cytoplasmic (outer) side would correspond to the inner side of the plasma membranes. A phospholipid distribution consistent with this view was also found for LM cell plasma membrane derivatives [5].

Sarcoplasmic reticulum is able to synthesize phosphatidylcholine de novo from diglyceride and CDPcholine [43]. The analogous enzyme in liver microsomes has been localized on the cytoplasmic surface of the membranes [44,45]. If this is also true for sarcoplasmic reticulum, newly synthesized phosphatidylcholine has to move transversely to be incorporated in the inner monolayer.

The exchange experiments suggest that at least 80% of the sarcoplasmic reticulum phosphatidylcholine is rapidly available for exchange from the outer surface (Fig. 5 and Table III). If 40% of the phosphatidylcholine is located in the outer monolayer, it follows that at least part of the phosphatidylcholine is involved in a rapid transbilayer movement (half-time <10 min). Our results are different from those of Bennett et al. [46], who quoted some unpublished observations which would indicate that only the phosphatidylcholine in the outer monolayer of sarcoplasmic reticulum vesicles is available for exchange and that transbilayer movement of this lipid is extremely slow.

Considering the mechanism of this rapid transbilayer movement of part of the phosphatidylcholine it could be speculated that this might occur via the approximately 20% of the lipid molecules that undergo a nearly isotropic motion in a fluid region of the membrane [47] in analogy with the rapid transverse movement of phospholipids in liver microsomes [23,48]. Alternatively, the intrinsic membrane proteins like the Ca^{2+}-ATPase itself might facilitate this transport as was reported for glycophorin [32,49].

A minor pool (less than 20%) of the phosphatidylcholine can be exchanged only very slowly (Fig. 5 and Table III). This pool can as yet not be localized in either outer or inner monolayer. This pool may be immobilized by intrinsic membrane proteins as has been proposed for sarcoplasmic reticulum Ca^{2+}-ATPase [50] and for cytochrome *c* oxidase [51].

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