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Reconstitution of $Ca^{2\oplus}$-ATPase of Sarcoplasmic Reticulum with ^{13}C-Labelled Lipids

^{13}C-NMR Spectroscopic Studies

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Summary: Sarcoplasmic reticulum of rabbit muscle has been isolated and delipidated. The enzymatically inactive apo-$Ca^{2\oplus}$-ATPase regained up to 50% of its original enzyme activity upon addition of $^{13}C/^{14}C$-labelled oleic or linoleic acid and lysolecithin. 90 to 180 fatty acyl chains were bound with reactivation of the $Ca^{2\oplus}$-ATPase, which obtained a density of 1.13 - 1.17 equal to that of native sarcoplasmic reticulum. No bilayer structure is formed. Only the firm binding of the fatty acid chains is required and no interactions with the polar head groups. This is expressed by a 60% reduction of the spin-lattice relaxation time (T_1-time) of free [14-^{13}C]linoleic acid and 2-[14-^{13}C]linoleoyllysolecithin. The [N-$^{13}CH_3$]choline head group of lysolecithin is not altered in its mobility after binding to the apo-$Ca^{2\oplus}$-ATPase.

A phospholipid exchange method is described, which allows an almost complete phospholipid exchange in the presence of sodium cholate. Cholate is completely removed by Sephadex G-25 chromatography and subsequent sucrose gradient centrifugation. The $Ca^{2\oplus}$-ATPase is enriched by this procedure. Proteins with molecular weights below 100 000 are removed. The native phospholipids of sarcoplasmic reticulum were exchanged against lecithins specifically labelled in their fatty acyl chains and the choline polar head groups without loss of enzymatic activity. 90 to 180 molecules were bound, which is twice the number of fatty acyl chains in the reactivation with free fatty acids and lysolecithins.

We conclude from the T_1-time measurements that only half of the fatty acids of the phospholipids are bound to the apoprotein, the rest forming the bilayer and being freely mobile in it.

The $Ca^{2\oplus}$-ATPase activity is twice to three times as high as in native sarcoplasmic reticulum when highly unsaturated lecithin species (dilinoleoylphosphatidylcholine) are used in the exchange method. $Ca^{2\oplus}$ translocation of these sarcoplasmic reticulum vesicles with highly unsaturated lecithins but depleted of phosphatidylethanolamine is very low. Exchange of less unsaturated lecithin species and phosphatidylethanolamines enhanced partially the $^{45}Ca^{2\oplus}$ storage.

Enzymes:
$Ca^{2\oplus}$-ATPase, ATP phosphohydrolase (EC 3.6.1.3);
Lactate dehydrogenase, L-lactate:$NAD^{\oplus}$ oxidoreductase (EC 1.1.1.27);
Phospholipase A_2, phosphatide 2-acylhydrolase (EC 3.1.1.4);
Pyruvate kinase, ATP:pyruvate 2-*O*-phosphotransferase (EC 2.7.1.40).

Abbreviations:
T_1-time = spin lattice relaxation time; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; SR = sarcoplasmic reticulum; EGTA = *O,O*-bis(2-aminoethyl)ethyleneglycol *N,N,N',N'*-tetraacetic acid.

Rekonstitution der $Ca^{2\oplus}$-ATPase des sarkoplasmatischen Retikulums mit ^{13}C-markierten Lipiden. ^{13}C-NMR spektroskopische Studien

Zusammenfassung: Sarkoplasmatisches Retikulum des Kaninchenmuskels wurde isoliert. Delipidierte und enzymatisch inaktive $Ca^{2\oplus}$-ATPase wurde durch $^{13}C/^{14}C$-markierte Öl- oder Linolsäure und Lysolecithin bis auf 50% der Ausgangsaktivität reaktiviert. Es wurden 90 bis 180 Fettsäureketten für die Reaktivierung gebunden. Diese so rekombinierte ATPase, deren Dichte (ρ = 1.13 - 1.17) mit der des nativen sarkoplasmatischen Retikulums übereinstimmt, liegt nicht als Doppelschicht vor, d.h. die $Ca^{2\oplus}$-ATPase Aktivität ist nicht an die Integration in eine Lipid-Doppelschicht gebunden. Für die Aktivierung der enzymatischen Aktivität ist die Bindung der Fettsäurekette verantwortlich. ^{13}C-NMR-Spin-Gitter-Relaxationszeitmessungen zeigen, daß die Beweglichkeit der Kohlenwasserstoffketten sowohl der freien Fettsäuren ([14-^{13}C]Linolsäure) als auch derselben Fettsäure im Lysolecithin gebunden nach der Bindung an die apo-$Ca^{2\oplus}$-ATPase um 60% reduziert wird. Die polare Gruppe ([*N*-$^{13}CH_3$]Cholin) verändert hingegen ihre Beweglichkeit durch die Bindung der Lysolecithinmoleküle nicht.

Es wird eine Phospholipid-Austauschmethode beschrieben, die in Gegenwart von Natriumcholat zum weitgehenden Austausch der Phospholipide führt. Durch Gelchromatographie an Sephadex G-25 und eine Rohrzuckergradientenzentrifugation (15 - 35% w/w) wird das Natriumcholat vollständig entfernt. Die $Ca^{2\oplus}$-ATPase wird in dieser Methode angereichert, die Proteine mit einem Molekulargewicht unter 100 000 werden abgetrennt. Unter Erhaltung der enzymatischen $Ca^{2\oplus}$-ATPase-Aktivität wurden Vesikeln des sarkoplasmatischen Retikulums mit in spezifischen Positionen der Fettsäureketten und der Cholingruppe ^{13}C-markierten Phospholipiden gebildet. Es wurden 90 bis 180 Lecithinmoleküle gebunden, d.h. die doppelte Anzahl von Fettsäuren wie bei der Fettsäure-Lysolecithin-Reaktivierung.

Aus den T_1-Zeit Messungen kann geschlossen werden, daß nur die Hälfte der Fettsäureketten der Phospholipidmoleküle an das Apoprotein gebunden wird, während die anderen in Doppelschicht-Struktur frei beweglich sind. Die $Ca^{2\oplus}$-ATPase-Aktivität der Vesikeln, die überwiegend das in der Austauschmethode eingesetzt hochungesättigte Phosphatidylcholin enthalten, ist zwei- bis dreimal so hoch wie im nativen sarcoplasmatischen Retikulum. Die $Ca^{2\oplus}$-Speicherung dieser nur das hochungesättigte Phosphatidylcholin aber kein Phosphatidyläthanolamin enthaltenden $Ca^{2\oplus}$-ATPase-Vesikeln ist sehr gering. Substitution mit weniger ungesättigten Lecithinspezies steigert die $^{45}Ca^{2\oplus}$-Speicherung.

Key words: Sarcoplasmic reticulum, phospholipid exchange, $Ca^{2\oplus}$-ATPase, [^{13}C]phospholipid relaxation studies, hydrophobic lipid-apoprotein interactions.

The calcium pump of sarcoplasmic reticulum (SR) is an easily accessible ATPase, which is being investigated actively in a number of laboratories[1-8]. This intrinsic membrane protein is particularly suited for studies on the interactions of the polypeptide chain with its bordering lipids since ATPase activity and pump properties can be correlated with the structure and physical properties of the phospholipids, mainly phosphatidylcholines and -ethanolamines forming the matrix of the SR membrane lipid bilayer.

Whereas the purification of the enzyme protein presents no obstacles, the reconstitution and integration of the ATPase into lipid vesicles is a more difficult matter.

In order to obtain further insight into the SR membrane structure, we initiated recombination studies of delipidated SR and purified ATPase and ^{13}C-labelled phospholipids. Spin-lattice relaxation time measurements of the reassembled phospholipid-enzyme complex, in which either the polar head group (*N*-CH_3 of choline) or the fatty acyl chains of the lipid were labelled in different carbon atoms (C-14, C-3), were carried out. Reactivation of $Ca^{2\oplus}$-ATPase was achieved with 1) [^{13}C]fatty acids and [^{13}C]lysolecithins, 2) [^{13}C]phospholipids.

In this publication we describe a phospholipid exchange procedure by which phospholipids of native SR are substituted by ^{13}C-labelled phospholipids. In comparative studies delipidated enzymatically inactive SR-ATPase was reactivated by [^{13}C]fatty acids and lysolecithin.

^{13}C-NMR spin lattice relaxation time experiments provide evidence for the hydrophobic interactions of the ATPase apoprotein with the fatty acyl chains of the phospholipids in the native SR.

Methods and Materials

^{13}C- and ^{14}C-labelled lipids: The synthesis of [14-^{13}C]-linoleic acid, 2-[14-^{13}C]linoleoyl-1-stearoyl-*sn*-glycero-3-phosphocholine, 2-[14-^{13}C;9,10,12,13-$^{3}H_4$]linoleoyl-*sn*-glycero-3-phospho-[*N*-$^{13}CH_3$]choline (lysolecithin) and [*N*-$^{13}CH_3$]lecithin and lysolecithin derived thereof has been described before[9,10]. The lecithin used was kindly provided by the Fa. Nattermann & Cie, Köln.

It is a highly unsaturated soyalecithin fraction of the following composition: 16:0 13.0%; 18:0 4.5%; 18:1 10.5%; 18:2 66.5%; 18:3 5.5% (1-position: 16:0 24%; 18:0 8.0%; 18:1 11%; 18:2 52.2%; 18:3 4.8%; 2-position: 16:0 2%; 18:0 1%; 18:1 10%; 18:2 80.5%; 18:3 6.5%).

Sodium [^{14}C]cholate was purchased from Buchler-Amersham, Braunschweig.

Phospholipase A_2, lactate dehydrogenase, $NAD^{\oplus}$, ATP and phospho*enol*pyruvate were purchased from Boehringer Mannheim GmbH.

Analytical procedures: Lipids were extracted according to Folch et al.[11]. Phospholipids were separated on thin-layer plates coated with a 0.25 mm Silica gel H film. They were activated for 2 h at 110 °C before use.

Radioactive bands were scanned with a radiochromatogram scanner Packard, model 7201, or thin-layer scanner II, Berthold LB 2722. Radioactive bands were eluted according to Goldrick et al.[12]. Fatty acid methyl esters were analyzed by gas chromatography on a Packard gas chromatograph model 805. 2-m columns with 15% and 2.5% ethylenglycol succinate polyester (EGS) on chromosorb were used.

Sodium dodecylsulfate gel electrophoresis in 4M urea was carried out according to Meissner et al.[13]. Test proteins were used as standards for protein determination.

Phospholipid phosphorus was determined according to Rouser[14].

Stoichiometry of lipid-apoprotein complexes was derived from a) specific activity of fatty acids ([1-^{14}C]palmitic, [9,10-$^{3}H_2$]oleic and [9,10,12,13-$^{3}H_4$]linoleic acid) and b) from the specific activity and microphosphorus determination of [*N*-$^{14}CH_3$]lysolecithin and the [^{3}H]- and [*N*-$^{14}CH_3$]lecithins after thin-layer chromatographic separation and quantitative recovery by established procedures[12].

SR protein was determined by the procedure of Lowry et al.[15]. The extinction values were correlated to the protein values determined by the Kjeldahl procedure[16] corrected for the lipid nitrogen.

Liposomes were prepared by ultrasonication with a Branson sonifier for 2 h, 75 Watt in a thermostat. The sonication vessel was flushed with argon during sonication. The vesicle preparation was centrifuged for 10 min at 1000 × *g* to remove titanium abrasions.

Electron micrographs: Negative staining was carried out with phosphotungstic acid prepared as a 2% solution adjusted to pH 6.5 with NaOH.

Freeze fracturing: Isolated SR was fixed for 2 h in Tris-buffered 2% glutaraldehyde (pH 7.4). After centrifugation at 100 000 × *g* for 30 min, the pellet was infiltrated with 25% glycerol in Tris buffer as a cryoprotectant before being placed on gold disks and frozen in liquid Freon 22 and then transferred to liquid nitrogen. The frozen SR was placed on the − 170 °C cold stage of a Balzers BA 360 M freeze-etch device, evacuated to 0.67 mPa (= 5×10^{-6} Torr), warmed to − 100 °C and fractured with a cold metal blade at − 170 °C, shadowed directly or after a 5 s etching time at 45 °C with platin carbon (20 Å) and coated from directly above with carbon (200 Å) to produce the replica by using an electron beam gun. The organic material was digested from the replica with sodium hypochlorite solution and the replicas were viewed in a Philips EM 300 electron microscope working at 80 kV.

Thin sectioning: Pellets were fixed in Tris-buffered 2% glutaraldehyde and after washing, postfixed in barbital-buffered 2% osmium tetroxide, pH 7.2, for 2 h. The pellets were alcohol dehydrated infiltrated with 1% Agar and embedded in a mixture of DDSA (dodecenylsuccinic anhydride)/Epon/Araldit M 2.2:1:0.8 using 1.7% 2,4,6-tris(dimethylaminomethyl)phenol (DMP 30) as a catalyst. All reagents were purchased from Serva, Heidelberg. Sections were cut on a LKB Ultramicrotome using glass knives and stained for 5 min with Reynold's lead citrate.

Enzyme assay: $Ca^{2\oplus}$-ATPase activity was measured in a combined optical test[17-19]. 5 µmoles NADH, 40 µl pyruvate kinase (8 U/ml) and 30 µl lactate dehydrogenase (8 U/ml) were added to 10 ml of a solution of: 2.5mM phospho*enol*pyruvate, 5mM ATP, 5mM $MgCl_2$, 0.5mM

EGTA, 20mM histidine and 0.1M KCl, pH 6.4. Each incubation mixture had a total volume of 2 ml. It was preincubated for 5 min at 37 °C in order to determine the $Ca^{2\oplus}$-independent ATPase activity. The reaction was started by the addition of 10 µl of a 0.123M $CaCl_2$ solution and the decrease of the absorption at 340 nm recorded continuously. $Ca^{2\oplus}$-ATPase activity is equal to the difference between total and $Ca^{2\oplus}$-independent activity.

^{13}C-NMR spectroscopy: Proton-decoupled ^{13}C Fourier transform NMR spectra were obtained at 25.2 MHz (14.2 kG) with a Bruker WH 90 spectrometer interfaced to a Nicolet BNC 12 computer. 20% D_2O was used for the lock. Partially relaxed Fourier transform (PRFT) spectra were obtained with the 180°-τ-90° pulse sequence[20]. τ value in the 180°-τ-90° sequence was chosen to bracket the inversion point at which $T_1 = \tau/\ln 2$. The T_1-times were computed from the plot $\ln(M_0 - M_z)$ vs τ, where M is the intensity of the peak. In repeated experiments the procedure was carried out with a standard error in the determination of 5 - 8% of the T_1-value. All samples were thoroughly flushed with argon; 10 mm sample tubes were used. Temperature was regulated with a Bruker ST 100/700 temperature control unit.

The *isolation and delipidation of native SR* was essentially carried out according to Hasselbach and Markinose[2]. 380 g muscle tissue yielded about 800 mg SR with a $Ca^{2\oplus}$-ATPase activity of 2 - 3 U at 37 °C. The sarcoplasmic reticulum was rapidly frozen and stored without loss of activity at − 190 °C for more than one month. For delipidation 50 mg SR (spec. act. 1.9 U) was pelleted at 100 000 × *g* for 20 min at 4 °C in a 60 Ti Beckman rotor. The pellet was homogeneously suspended in 15 ml of a 0.1M KCl, 0.02M histidine, 5mM $CaCl_2$ buffer pH 7.0, 0.5 mg phospholipase A_2 (200 U/mg) dissolved in 0.5 ml of the same buffer was added and the mixture stirred for 2 - 3 h at 4 °C.

The progress of the phospholipid hydrolysis was followed by thin-layer chromatography (solvent system: chloroform/methanol/water 65:25:4). The activity was 1.6 U/mg at this point. The SR was pelleted by centrifugation at 100 000 × *g* for 30 min at 4 °C. The pellet was washed twice with 25 ml of a 5% delipidated (acetone washed) bovine serum albumin solution for 10 min at 4 °C. The delipidated SR was sedimented at 100 000 × *g* 30 min and the residual albumin washed out of the pellet twice with 25 ml 0.1M KCl. After this procedure the delipidated SR $Ca^{2\oplus}$-ATPase activity was 0.24 U. The yield of apoSR was 40 mg (80%).

Reactivation of $Ca^{2\oplus}$-ATPase with fatty acid and lysolecithin. General Procedure: Micelles of oleic or linoleic acid (31 µmol) and 1-palmitoyl-*sn*-glycero-3-phosphocholine (34 µmol) in 100 ml recombination buffer (0.02M histidine; 0.1M KCl, 5mM $MgCl_2$, pH 8.4) were prepared by ultrasonication at 0 °C and a power setting at 75 W of the Branson sonifier. 27 mg of apoSR suspended in 2 ml recombination buffer was added to the micellar lipid solution and stirred for 30 min at 4 °C. The reactivated SR (spec. act. 1.0 U/mg) pelleted at 100 000 × *g*, 45 min. The pellet was suspended in the histidine buffer pH 7.4 for sucrose gradient (26 - 41% w/w) centrifugation. This centrifugation (SW 27 rotor at 27 000 rpm, 4 °C) lasted 13 h. The recombined SR banded at a density of 1.13 - 1.17 g/ml. Non-recombined apoSR sedimented. The reactivated SR was diluted with the buffer and sedimented at 100 000 × *g*, 45 min. For ^{13}C-NMR experiments the pellet was homogenized in 0.1M KCl, 0.005M $MgCl_2$, 0.1% NaN_3. Under these conditions about 40 to 50% of apoSR was relipidated and reactivated.

Exchange of phospholipids of native sarcoplasmic reticulum against defined phospholipid species

Rabbit skeletal muscle sarcoplasmic reticulum was prepared following the procedure outlined by Hasselbach and Makinose[2]. The native SR (25 mg protein/ml) was suspended in the following buffer: 0.25M sucrose, 10 mmol Tris/HCl, pH 8.0.

60 mg (80 µmol) of the lecithin species used and 60 mg (145 µmol) sodium cholate were sonicated in 24 ml of buffered sucrose solution (0.25M sucrose, 0.025M potassium phosphate buffer, pH 8.0, 1M KCl) for 10 min with cooling in an ice bath using the microtip of a Branson sonifier at an output of 75 W. The clear solution was added to the above SR suspension and stirred at 4 °C for 1 h. The slightly opalescent mixture was passed over a Sephadex G-25 column (2.5 × 60 cm) and a Tris buffer (0.05M Tris, 0.4M KCl, 1 mmol histidine, 5 mmol $MgCl_2$, 0.1 mmol $CaCl_2$, 1 mmol EDTA, 0.01% NaN_3, pH 7.3) was used for the elution. Fractions of 10 ml were taken. The turbid SR with the exchanged phospholipids eluted in the front. Sodium cholate separated completely from this band, as checked with ^{14}C-labelled sodium cholate.

The SR sedimented on centrifugation at 38 000 rpm in a 60 Ti rotor for 45 min at 15 °C. The pellet was suspended with a pasteur pipette in the same buffer. The suspension was layered on top of a buffered sucrose gradient (15 to 35% w/w) in the Tris buffer, pH 7.3, used for Sephadex G-25 chromatography. Centrifugation at 35 000 rpm for 18 h at 4 °C in a SW 41 Beckman rotor yielded the SR-band at ρ = 1.120 - 1.150 g/ml.

Whenever ^{13}C-labelled lipids were used for NMR experiments the SR pellet was suspended in the following buffer: 0.05 mmol NH_4HCO_3, 0.4M KCl, 5 mmol $MgCl_2$, 0.01% NaN_3, 20% D_2O, pH 7.3.

The degree of phospholipid exchange was measured by chloroform/methanol (2:1) extraction of the total lipids, their thin-layer chromatographic separation chloroform/methanol/water 65:25:4), transesterification of the lipids with 5% methanolic HCl, and subsequent gas-chromatographic analysis of fatty acid methyl esters by standard procedures.

Results

1) Reactivation of SR $Ca^{2\oplus}$-ATPase by [14-^{13}C]linoleic acid and 2-[14-^{13}C]linoleoyllysolecithin

Rabbit skeletal muscle SR was prepared according to Hasselbach and Makinose[2] and delipidated after phospholipase A_2 treatment by washing with serum albumin as described by Fiehn

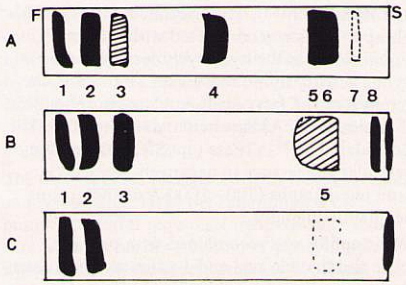
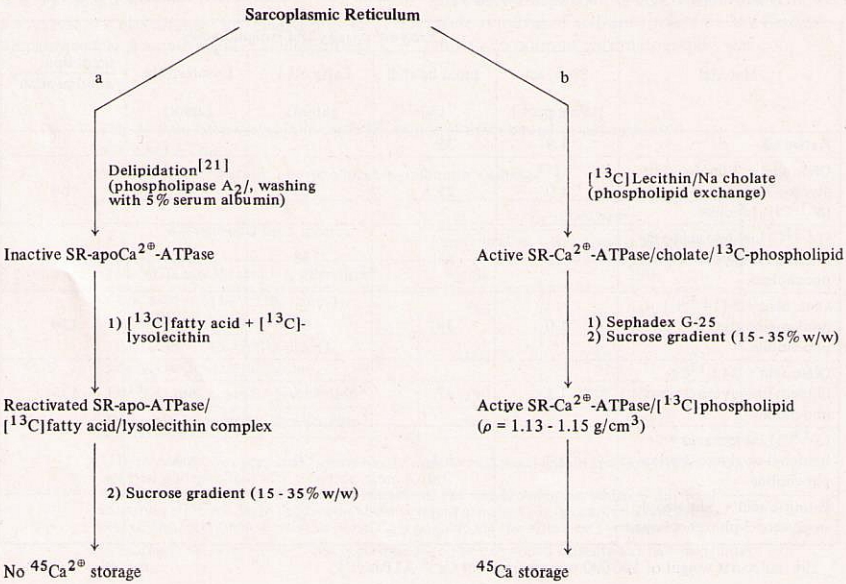


Fig. 1. Thin-layer chromatographic separation of lipids of A) native SR, B) after treatment with phospholipase A_2, C) after removal of fatty acids and lysolecithin by washing with bovine serum albumin.
1 = triglyceride, 2 = cholesterol, 3 = fatty acids, 4 = phosphatidylethanolamine, 5 = glycerol, 6 = lecithin, 7 = sphingomyelin, 8 = lysolecithin, F = front, S = start. Solvent system: chloroform/methanol/water 65:25:4.

Table 1. Flow sheet of a) delipidation and reactivation of SR-$Ca^{2\oplus}$-ATPase and b) phospholipid exchange procedure of SR.



and Hasselbach[21]. The enzymic hydrolysis of phospholipids was completed within 2 to 3 h, Fig. 1. Whereas the hydrolysis decreased the enzymic activity by no more than 10 to 15%, the removal of the fatty acids and lysophospholipids diminished the ATPase activity to 5 to 10%. The delipidated $Ca^{2\oplus}$-ATPase (apoSR) retained the vesicular appearance in negatively stained electron micrographs (700 - 1000 Å diameter) but sedimented rapidly.

When apoSR was recombined with palmitic, oleic and linoleic acid and 1-palmitoyl-*sn*-glycero-3-phosphocholine in a histidine buffer, pH 8.4, a 2×10^{-4}M concentration was optimal. The procedure is summarized in Table 1 and the restoration of $Ca^{2\oplus}$-ATPase activity in Table 2. Palmitate was unable to reactivate the $Ca^{2\oplus}$-ATPase assayed under the conditions used throughout these studies (37 °C), but the unsaturated fatty acids together with saturated or unsaturated lysolecithins led to a considerable (about 50%) reactivation, Table 2. This table also summarizes the stoichiometry of the reactivated SR-lipid complex. The reactivated ATPase complex banded in a sucrose gradient (15 to 35% w/w) at a density of 1.15 - 1.17 at 20 °C, the native SR at ρ = 1.13 - 1.17 g/ml[22]. These procedures led to a considerable enrichment of the ATPase, Fig. 2. The reactivation was performed with ^{13}C-labelled linoleic acid and lysolecithin, and the spin lattice relaxation times (T_1) of these complexes were measured and compared with those of the micellar forms of these lipids, Table 3.

It is apparent that the [*N*-$^{13}CH_3$]choline group of the lysolecithin is freely mobile, whereas considerable restrictions of the mobility of the carbon chains of the unsaturated fatty acids are noticed. The ^{13}C-NMR spectra exhibit resonances of very different shape. The sharp signal of the *N*-$^{13}CH_3$ group of lysolecithin at 54 ppm (Fig. 3a) refers to its rather unrestricted motions,

Tab. 2. Reactivation of apo$Ca^{2\oplus}$-ATPase of sarcoplasmic reticulum by ^{13}C-labelled fatty acids and lysolecithin.

Material	Enzyme activity and stoichiometry				
	Spec. act. [U/mg prot.]	Lipid bound [%]	Fatty acid [μmol]	Lysolecithin [μmol]	$\frac{\mu\text{mol lipid}}{\mu\text{mol protein}^*}$
Native SR	1.9	35	–	–	
Oleic acid + 2-linoleoyl-*sn*-glycero-3-phospho-[*N*-$^{13}CH_3$]choline	1.0	25.5	40	48	88
[14-^{13}C]Linoleic acid + 2-linoleoyl-*sn*-glycero-3-phosphocholine	1.3	n.d.	138	n.d.	
Oleic acid + 2-[14-^{13}C]-linoleoyl-*sn*-glycero-3-phosphocholine	1.0	38	60	70	130
Oleic acid + 2-[3-^{13}C]-linoleoyl-*sn*-glyero-3-phosphocholine	1.1	37	115	60	175
[3-^{13}C]Linoleic acid + 2-linoleoyl-*sn*-glyero-3-phosphocholine	1.3	n.d.	30	n.d.	
Palmitic acid + 2-linoleoyl-*sn*-glycero-3-phosphocholine	0	–	–	–	–

* The molecular weight of 100000 was assumed for $Ca^{2\oplus}$-ATPase[3].

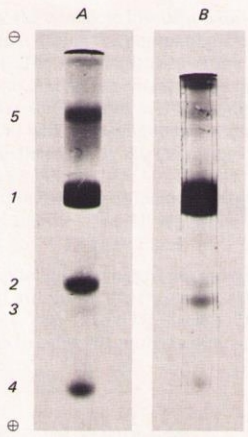


Fig. 2. Polyacrylamide (6%) gel electrophoresis in the presence of 1% Na dodecylsulfate and 4M urea (system 2 of ref.[113]) of A) protein of native SR, B) delipidated SR, recombined with linoleic acid and lysolecithin and banded by sucrose gradient centrifugation (15 - 35% w/w).

1 = ATPase, 2 = high affinity $Ca^{2\oplus}$ binding protein, 3 = calsequestrin, 4 = proteolipid, 5 = unidentified.

whereas the resonances of the [14-^{13}C]linoleic acid (Fig. 3b) bound as free fatty acid or in 2-linoleoyl-*sn*-glycero-3-phosphocholine (prepared by the action of triacylglycerol lipase on 1,2-[14-^{13}C]dilinoleoyl-*sn*-glycero-3-phosphocholine) are considerably broader due to their immobilization.

The $Ca^{2\oplus}$-ATPase reactivated with free fatty acids and lysolecithin lacks the function of a $Ca^{2\oplus}$-pump. The latter requires a tight vesicular structure with the barrier function of phospholipids, which is lost in the reactivated $Ca^{2\oplus}$-ATPase.

2) A method for the exchange of phospholipids of native SR against ^{13}C-labelled lecithins

The lipid pool of the SR was exchanged with a mixture of a given lecithin species and sodium cholate (1:1.6, molar ratio) (see Table 1 and Methods and Materials). A 300-fold molar excess of the lecithin species was used, taking the molecular weight 100 000 for $Ca^{2\oplus}$-ATPase as the basis of the calculation. Sodium cholate was then completely removed from the opalescent solution by passing the SR-lecithin-cholate complex over a Sephadex G-25 column. The complete removal of sodium cholate by the Sephadex G-25 column chromatography was con-

Tab. 3. Spin-lattice relaxation times (T_1 in ms) of lipids bound to sarcoplasmic reticulum ATPase.
Method of delipidation: phospholipase A_2/albumin washings[12].

Recombined lipid species	T_1-times [ms]	
	Lipid micelles	Reactivated SR complex
[14-^{13}C]Linoleic acid + lysolecithin*	681	290
Oleic acid + 2-[14-^{13}C]linoleoyl-*sn*-glycero-3-phosphocholine	786	280
Oleic acid + 2-[3-^{13}C]linoleoyl-*sn*-glycero-3-phosphocholine	565	$T_2^* = 8 \times 10^{-4}$
[3-^{13}C]Linoleic acid + lysolecithin*	290	$T_2^* = 10^{-3}$
Oleic acid + [*N*-$^{13}CH_3$]lysolecithin*	565	590

* Lysolecithin was prepared from a highly unsaturated soyalecithin fraction (EPL, essential phospholipid of Fa. Nattermann, Köln)
** T_1-times could not be measured because of the line broadening, which is due to the immobilization of the hydrocarbon chain segment around C-3. An approximate estimation of the rotational correlation time T_c is possible via the effective T_2-time measurement. $\Delta V = \frac{1}{\pi \times T_2}$. ΔV corresponds to the line width measured at half maximum intensity.

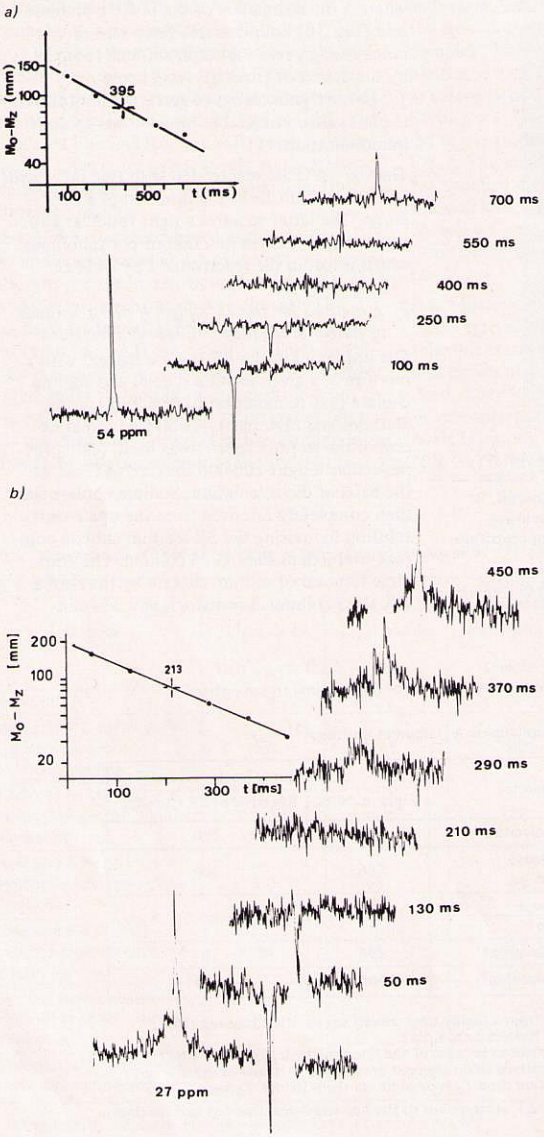


Fig. 3. ^{13}C-NMR spectrum and T_1-determination of a) [N-$^{13}CH_3$]lysolecithin and oleic acid bound to delipidated SR, b) [14-^{13}C]linoleic acid and lysolecithin.

firmed by the complete loss of sodium [^{14}C]-cholate used during the phospholipid exchange procedure. The specific activity of the SR $Ca^{2\oplus}$-ATPase was 2 U before cholate removal. The cholate-free $Ca^{2\oplus}$-ATPase-lecithin complex eluted in the front with excess unbound phospholipid and was sedimented by ultracentrifugation (100 000 × *g*, 45 min, 15 °C). The sediment was suspended in buffer for gradient centrifugation (15 - 35% sucrose). The SR sedimented at a density ρ = 1.15 - 1.17 g/ml, Fig. 4.

The phospholipid exchanged SR showed up to a three-fold increase in the enzymatic activity of the $Ca^{2\oplus}$-ATPase (3 to 6 U), Table 4. Only faint bands due to other SR proteins were visible in

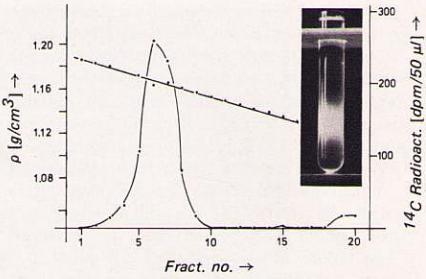


Fig. 4. Sucrose gradient (15 - 35% w/w) centrifugation of apoSR associated with exchanged 1,2-[14-^{13}C]-dilinoleoyl-*sn*-glycero-3-phosphocholine.

Tab. 4. Spin-lattice relaxation times (T_1 in ms) of lipids, stoichiometry and enzymatic activity of sarcoplasmic reticulum ATPase after phospholipid exchange.
Phospholipid – cholate exchange method.

Exchanged phospholipid species	T_1-times [ms]		Molar lipid/protein* ratio	Spec. act. [U/mg prot.]
	Lipid vesicles	ATPase-phospholipid complex		
1-Stearoyl-2-[14-^{13}C]linoleoyl-*sn*-glycero-3-phosphocholine	640	380	90:1	5.9
1,2-Di[14-^{13}C]linoleoyl-*sn*-glycero-3-phosphocholine	780	620	160:1	3.3
[*N*-$^{13}CH_3$]Lecithin**	510	480	180:1	5.5

* A molecular weight of 100000 was taken for the main protein, the $Ca^{2\oplus}$-ATPase.
** Soyalecithin (70% linoleic acid).

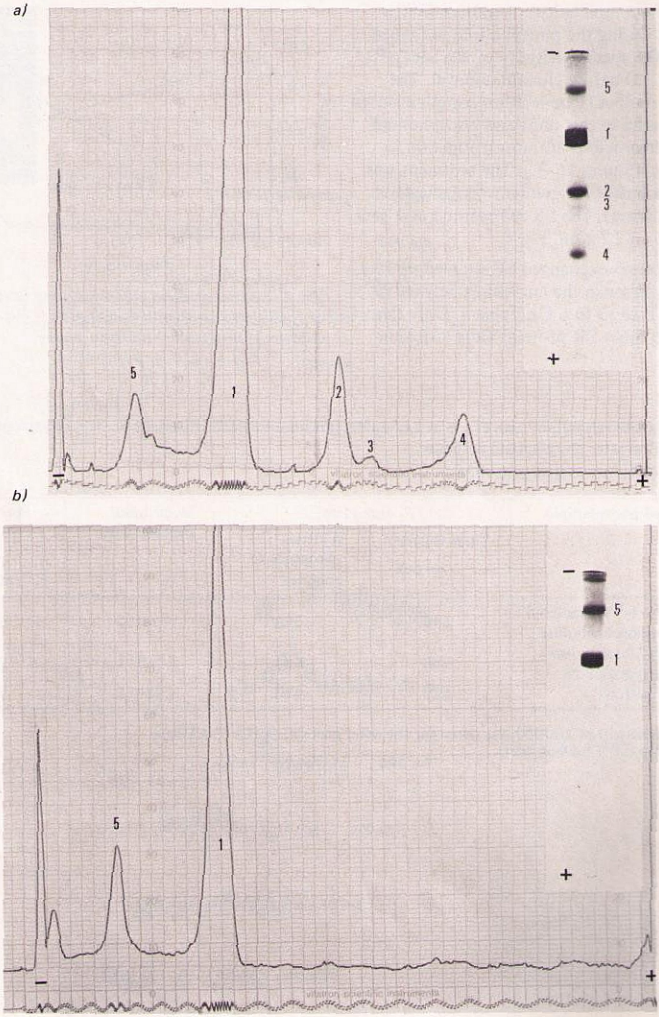


Fig. 5. Polyacrylamide gel (6%) electrophoresis in the presence of 1% Na dodecylsulfate and 6M urea (system 2 of ref.[13]) of a) native sarcoplasmic reticulum, b) SR after phospholipid exchange procedure.
1 = $Ca^{2\oplus}$-ATPase, *2* = high affinity $Ca^{2\oplus}$ binding protein, *3* = calsequestrin, *4* = proteolipid, *5* = unidentified protein.

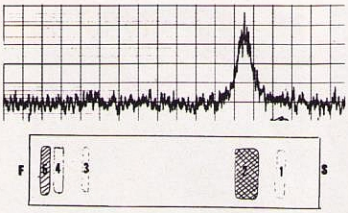


Fig. 6. Radio thin-layer chromatographic analysis of lipids of SR.

Phospholipids were exchanged against radioactive dilinoleoyl-*sn*-glycero-3-phosphocholine by the procedure described in the text. 1 = traces of lysolecithin, 2 = phosphatidylcholine, 3 = fatty acids, 4 = cholesterol, 5 = triglycerides, F = front, S = start. Solvent system: chloroform/methanol/water 65 : 25 : 4.

dodecylsulfate gel electrophoresis, Fig. 5 a, b. The analysis of the phospholipids finally bound to this $Ca^{2\oplus}$-ATPase preparation was carried out as follows: [*N*-^{14}C]choline labelled lecithin was admixed with the ^{13}C-labelled lecithin for exchange. Specific radioactivities (dpm/μmol phosphorus) of the lecithin extracted before and after the exchange indicated that more than 80% of the phospholipids of the native SR had been substituted by the labelled lecithin species. Thin-layer chromatographic analysis proved that phosphatidylethanolamine present at about 13.5% of total phospholipids[23] in native SR has completely disappeared, Fig. 6. Transesterification of the total exchanged lipid of SR, the lipid of which had been substituted by di-[14-^{13}C]linoleoyl-*sn*-glycero-3-phosphocholine, yielded linoleic acid as the main component (about 80%) and traces of palmitic, oleic and stearic acid, Fig. 7.

The electron microscopic appearance of the phospholipid-exchanged SR is that of vesicles of different diameter. They exhibit knob-like structures protruding from the outer surfaces of the negatively stained preparations, Fig. 8. The freeze fracture technique applied to native and phospholipid exchanged SR vesicles visualizes granular structures at the outer concave surface of the vesicle, but only a few on the inner convex surface (Fig. 9). Figures 9 a - c visualize the differences in the number of granular structures protruding from

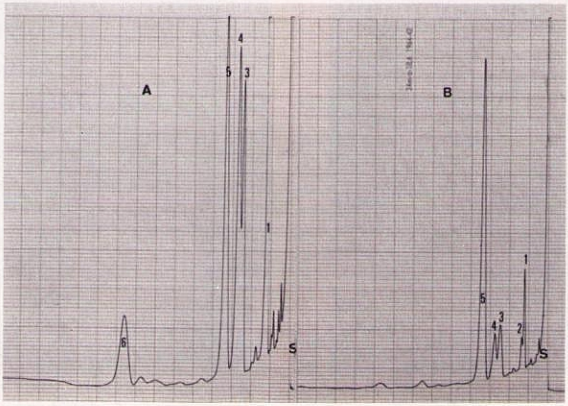
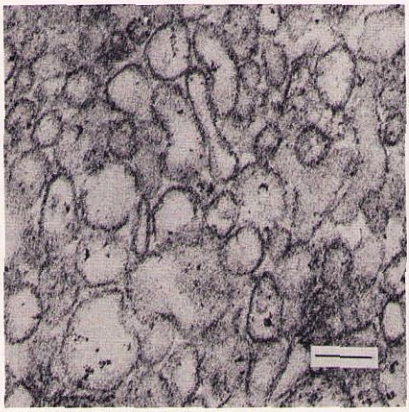
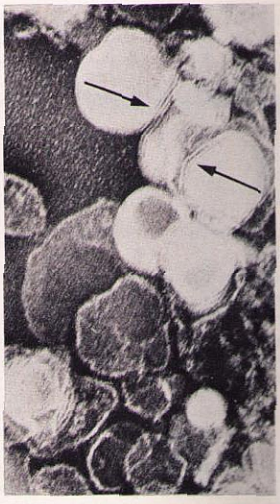


Fig. 7. Gas-chromatographic analysis of fatty acid methyl esters isolated from lecithin bound to native SR (A) and after exchange against di-[14-^{13}C]linoleoyl-*sn*-glycero-3-phosphocholine (B).

The lecithin fraction was isolated, purified and transesterified. 1 = 16 : 0, 2 = 16 : 1, 3 = 18 : 0, 4 = 18 : 1, 5 = 18 : 2, 6 = 20 : 4, S = start.

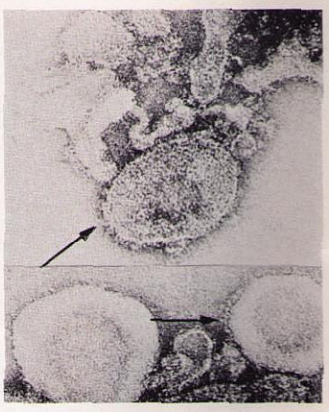


a) x 72000



b) x 137000

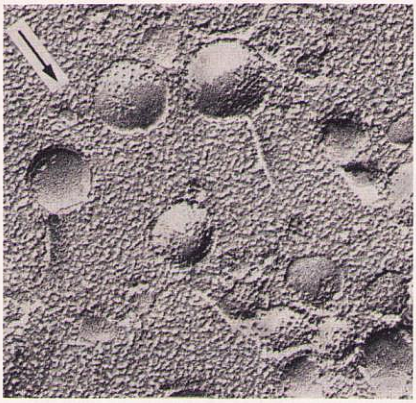
c) x 47000



x 137000

Fig. 8. Electron micrograph of ATPase vesicles containing dilinoleoyl-*sn*-glycero-3-phosphocholine as phospholipid species after negative staining, a) native SR (ultra thin section). Calibration bar in the lower right corner of the electron micrograph corresponds to 1250 Å; b) before sucrose gradient centrifugation (*note the multilamellar structures* of liposomes); c) after sucrose gradient centrifugation, in two different magnifications (note the knobs at the outer surface of the vesicles).

a)



b)

c)

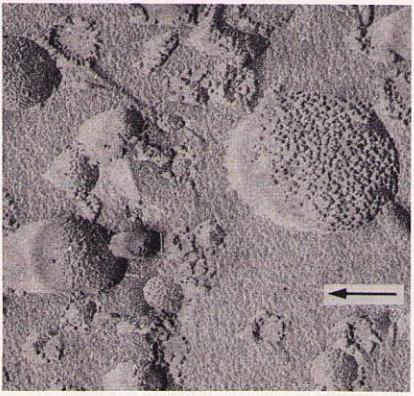


Fig. 9. Freeze fracture electron microscopy of a) sarcoplasmic reticulum (SR) vesicles, b) SR after phospholipid exchange against lecithin, c) SR reassembled with phospholipids according to Meissner et al.[13].
The bar serves the calibration (1250 A) and indicates the direction of platinum shadowing. Magnification x 72 000.

the replica of the convex and concave surfaces of vesicles resembling the ATPase in different preparations. These surfaces correspond to the inner and outer layers, respectively, of the bimolecular leaflet structure of the vesicles. Fig. 9a is the freeze fracture electron micrograph of native SR with vesicles of concave fracture faces with many particles, whereas the convex fracture faces are almost free of particles. The statistical analysis indicates a 9.6 : 1 ratio of particles in outer to inner shell of native SR vesicles (see Table 5). ATPase vesicles, Fig. 9b, obtained by our phospholipid exchange method exhibit a slight randomization of the pump protein over the outer and inner bilayer (6 : 1) with lecithin as single exchanged phospholipid, but 3.5 : 1 when a lecithin/phosphatidylethanolamine 1 : 1 mixture was exchanged against the native phospholipids

Tab. 5. Statistical evaluation of number of particles in outer (concave) and inner (convex) shell of sarcoplasmic ATPase preparations.
Particles were visualized by the freeze fracture technique (see Methods and Materials).

Preparation	Area [μ^2]		No. of particles	Particles/μ^2	No. of particles concave/convex
Native SR vesicles	concave	0.40	584	1460	9.6
	convex	0.41	62	151	
PC exchanged	concave	0.59	639	1083	6.12
	convex	0.52	92	177	
PC/PE (1:1) exchanged	concave	0.51	799	1567	3.5
	convex	0.55	248	451	

of SR. For comparison, the reassembly procedure of Meissner, Conner and Fleischer[13] was also used, Fig. 9c. A distribution of particles in the outer and inner half of the vesicle bilayer in a ratio of 1:1 was obtained.

For the study of the $Ca^{2\oplus}$-ATPase-phospholipid interactions by ^{13}C-NMR spectroscopy three lecithin species labelled with ^{13}C in specific positions were exchanged for the native phospholipids of SR, the mobility of the polar head group of the lecithin molecules was probed by [*N*-$^{13}CH_3$]lecithin, the fatty acid composition of which is given under Methods and Materials. No significant alteration of the T_1-time of this group was observed in the spin-lattice relaxation time experiment, Table 4. However, when 2-[14-^{13}C]linoleoyl-1-stearoyl- and 1,2-di-[14-^{13}C]linoleoyl-*sn*-glycero-3-phosphocholine were used in the phospholipid exchange, a considerable reduction of the T_1-times by 41 and 20%, respectively, of that of the same lecithins in liposomes was observed. The T_1-time experiment is recorded in Fig. 10. These results reflect the similar behaviour of the free fatty acid and lysolecithin; the polar head groups of which also remain unaltered with respect to their mobility, whereas the unsaturated fatty acyl chains are considerably immobilized by their interaction with the apoproteins.

Discussion

$Ca^{2\oplus}$-ATPase of skeletal muscle is an integral membrane protein of the sarcoplasmic reticulum, which translocates $Ca^{2\oplus}$ into the sarcoplasmic tubular system from the sarcoplasm with an ATP-driven pump. The $Ca^{2\oplus}$-ATPase is the main protein of SR, which is available in large quantity. Native SR and purified $Ca^{2\oplus}$-ATPase are very suitable to study energy transduction correlated to the membrane structure or apoprotein-lipid interactions. Fiehn and Hasselbach[21] demonstrated the dependence of enzymic activity and lipid requirement. Recent work by MacLennan et al. [24-27] and Meissner and Fleischer[4] led to the isolation of five proteins of SR: the major one, ATPase, (102 000 daltons), calsequestrin (44 000 daltons), the 30 000 dalton protein and the proteolipid of molecular weight 6 000. These proteins are associated with 35% lipid on a weight basis. There are 90 lipid molecules/ATPase molecule, 93% of which are phospholipids (73% phosphatidylcholine, 13.5% phosphatidylethanolamine, 9% phosphatidylinositol and traces of phosphatidylserine, sphingomyelin and cardiolipin)[4].

The mechanism by which the ATP-driven $Ca^{2\oplus}$ translocation occurs has been studied extensively[28-32]. The role of lipids in this process is being investigated in a number of laboratories in reconstitution experiments.

The insolubility of the apoATPase and the phospholipids requires for recombination studies either ultrasonication as applied by Racker[23] or detergents. Sodium deoxycholate was used by Meissner and Fleischer[34], cholate by Warren et al.[5] and Racker[23] and Triton X-100 by Walter

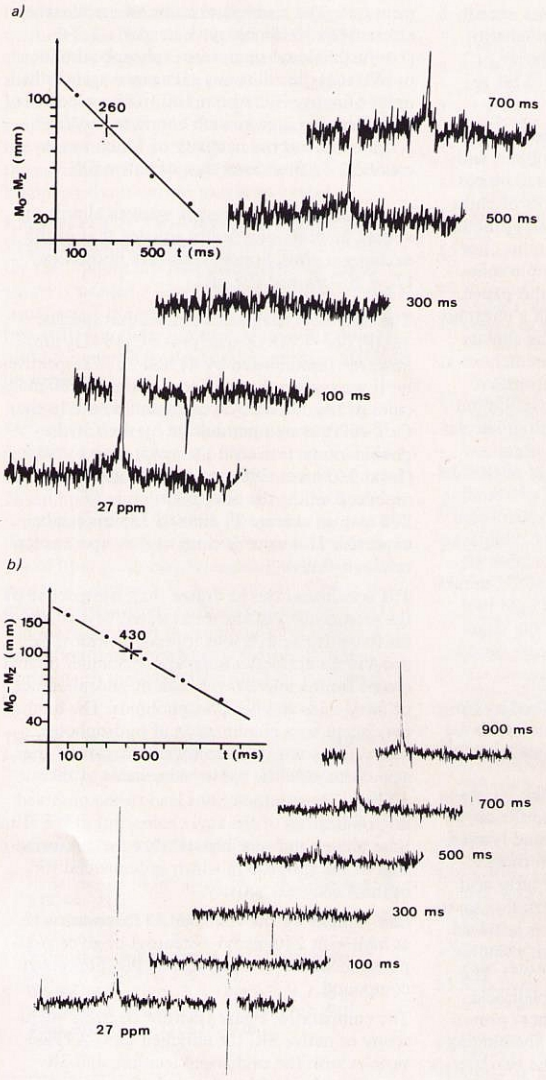


Fig. 10. Spin-lattice relaxation time (T_1) determination of a) 1-stearoyl-2-[14-^{13}C]linoleoyl-*sn*-glycero-3-phosphocholine and b) di-[14-^{13}C]linoleoyl-*sn*-glycero-3-phosphocholine assembled with rabbit sarcoplasmic $Ca^{2\oplus}$-ATPase by the phospholipid exchange method.

and Hasselbach[35,36]. Both detergents were removed by dialysis or a sedimentation substitution. The latter procedure allowed Warren et al.[37] to substitute the native lipids of SR to above 95%.

We have tried to apply this method to the recombination of ^{13}C-labelled phospholipids with SR-$Ca^{2\oplus}$-ATPase. However, it proved to be not suitable, mainly because large amounts of phospholipids are required to obtain suitable amounts of reconstituted enzyme for NMR studies. For these reasons we have elaborated the phospholipid exchange method described in this paper. It gives reproducibly high yields, with a phospholipid exchange of more than 80%. The cholate which mediates this exchange is completely removed by a rapid Sephadex G-25 chromatography, as proven by use of radioactive sodium cholate as detergent. All proteins with molecular weight below that of the $Ca^{2\oplus}$-ATPase are removed by this procedure (proteolipid, 30 000 dalton protein, M 55 and high affinity $Ca^{2\oplus}$ binding protein). The reconstituted enzyme substituted with the desired phospholipid species exhibits an enzymic activity higher than that of native SR. Depending on the lipid composition, $Ca^{2\oplus}$-uptake can also be restored in part. We have used two approaches in order to decide about the structural requirements of the lipid molecule for optimal $Ca^{2\oplus}$-ATPase activity.

The procedure of delipidation described by Hasselbach and Makinose[2] was combined with the reactivation by free fatty acids and lysolecithin.

The alkyl chains of both contained the ^{13}C-nucleus enriched in position 14, beyond the *cis*-double bond system of linoleic acid and lysolecithin and also in the choline group. In these recombination experiments 90 to 180 fatty acyl chains bind to the enzyme and reactivation up to 50% of the original enzyme activity is achieved. These fatty acyl chains must be fluid, a finding which agrees with previous reports[36,37]. The mutual exchange of the [14-^{13}C]linoleic acid – free or incorporated in lysolecithin – proved that irrespective of the polar group, the binding of the hydrophobic acyl chains of the two species led to the same immobilization of about 50%. These two lipids do not allow the formation of a lipid vesicle and therefore no $Ca^{2\oplus}$-storage can be measured. The same observation was made when either 2-[14-^{13}C]linoleoyl-1-stearoyl-, 1,2-di-[14-^{13}C]linoleoyl-*sn*-glycero-3-phosphocholine or [*N*-$^{13}CH_3$]lecithin was exchanged against the lipids of native SR. About 100 - 180 molecules of either lecithin species were bound to the $Ca^{2\oplus}$-ATPase, the enzymic activity of which was increased 2 - 3 times over that of native SR.

The choline group possessed again an almost unaltered mobility and nearly equaled that of the exchanged phospholipid species in liposomes (T_1 = 507 ms and 478 ms).

The fatty acyl chains of both lecithin species, 18:0/[14-^{13}C]18:2 and ([14-^{13}C]18:2)$_2$ are, however, immobilized by 41 and 22%, respectively. If we assume that 180 acyl chains of 180 molecules of the dilinoleoyl compound bound to the $Ca^{2\oplus}$-ATPase are immobilized by their hydrophobic interaction with the apoprotein by 50% (from 780 ms to 390 ms), but 180 are freely mobile, forming the bilayer structure (T_1 = 780 ms), an average T_1-time of 585 ms can be expected. This value is close to the experimental result (610 ms).

The conclusion can be drawn that, irrespective of the arrangement of the unsaturated acyl chains (as free fatty acid, lysolecithin or lecithin), the apoATPase extracts a comparable number of acyl chains from a micellar solution of phospholipids or fatty acids and lysophospholipids. The binding may occur by a combination of hydrophobic interactions and steric complementation of the acyl chains and the apolar side chains of the ATPase. These interactions lead to the observed immobilization of the acyl chains, but at the same time induce and possibly stabilize the conformation of the apoprotein which is demanded for optimal enzymic activity.

$Ca^{2\oplus}$-uptake by the vesicular ATPase was twice as high with 2-linoleoyl-1-stearoyl-*sn*-glycero-3-phosphocholine as compared to the dilinoleoyl compound.

The comparative freeze fracture electron microscopy of native SR, the enriched $Ca^{2\oplus}$-ATPase vesicles with the exchanged lecithin, and SR treated with a lecithin-deoxycholate mixture followed by removal of the detergent by exhaustive dialysis according to Meissner et al.[13]

indicates that our phospholipid exchange method leads only to a limited randomization of the ATPase granulas protruding from the outer shell of the vesicles. They are also recognizable as knoblike structures on negative staining of the preparation. Whatever the molecular event during the phospholipid exchange in our procedure is, the asymmetric arrangement of the $Ca^{2\oplus}$-ATPase is apparently preserved to a large extent.

Knowles et al.[38] postulate that lecithin as a single phospholipid class is unable to reactivate the $Ca^{2\oplus}$-pump but that phosphatidylethanolamine is needed.* The question of whether the structure of the acyl chains alone or in conjunction with specific polar head groups is a prerequisite for an efficiently working pump remains to be answered.

A general conclusion can be drawn from the ^{13}C-NMR studies. This method proved to be a valuable tool in this and previous studies for probing hydrophilic and hydrophobic interactions between lipids and proteins. However, the limitations also become clear. A favourable protein to lipid ratio of the system with a considerable number of lipid molecules interacting with the apoprotein is required. If, however, the lipid is predominantly involved in bilayer formation, this will inevitably lead to randomized spin-lattice relaxation times, abolishing discrete T_1-alterations due to the interaction of lipid molecules.

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* Present studies are concerned with the structural requirements for the fatty acyl chains and the polar head group (Stoffel, W. & Amoneit, T. to be published).

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