Biochimica et Biophysica Acta, 645 (1981) 124–131
Elsevier/North-Holland Biomedical Press

BBA 79255

STRUCTURAL AND FUNCTIONAL LABILITY INDUCED BY DIETHYL ETHER ON THE SARCOPLASMIC RETICULUM MEMBRANE

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(Received November 24th, 1980)
(Revised manuscript received February 18th, 1981)

Key words: Sarcoplasmic reticulum; Diethyl ether; Ca^{2+} transport; Phosphoenzyme turnover; Membrane fragility; (Rabbit skeletal muscle)

Structural and functional changes occurring in sarcoplasmic reticulum vesicles following exposure to low concentrations (5–7%, v/v) of diethyl ether in aqueous media, were studied by electron microscopy and by kinetic measurements of Ca^{2+} transport and ATPase activity. Electron microscopy of thin sectioned and freeze-fractured sarcoplasmic reticulum vesicles provided detailed resolution of Ca-ATPase amphiphilic molecules displaying 'lollipop' portions on the outer surface of the vesicle, and non-polar moieties penetrating the membrane's hydrophobic interior. This asymmetric disposition of ATPase molecules was disrupted in vesicles exposed to ether and then centrifuged and/or resuspended in aqueous media. Such vesicles had a tendency to undergo fragmentation, and the distribution of ATPase molecules was markedly altered. The continuous fuzzy layer of lollipops became discontinuous, and the intramembranous particles became randomly distributed over both the concave and the convex freeze-fracture membrane faces. Functionally, the vesicles lost their ability to accumulate calcium in the presence of ATP, although high rates of ATPase activity were maintained. Vesicles which were simply exposed to ether, without being subjected to centrifugation and/or homogenization, did not appear altered ultrastructurally, and retained their ability to accumulate calcium. In fact, the enzyme turnover and the maximal levels of calcium uptake were increased. It is concluded that diethyl ether interferes with lipid-lipid and protein-lipid interactions in the sarcoplasmic reticulum vesicle membrane, thereby facilitating molecular motions which may be a limiting factor in the transport mechanism. On the other hand, these weakened interactions permit structural denaturation and loss of the ability to maintain a transmembrane Ca^{2+} gradient when the vesicles are subjected to mechanical perturbations which are harmless in the absence of ether.

Introduction

Treatment with diethyl ether is one of the expedients used to render sarcoplasmic reticulum vesicles leaky, and to prevent net calcium accumulation in the presence of ATP [1,2]. Using this treatment, it is possible to prolong the ATPase burst which normally lasts only a few seconds following the addition of ATP and declines when the vesicles are filled with calcium. The ether-treated vesicles are a useful experimental system since they catalyze ATP hydrolysis at constant velocities, permitting studies of steady-state ATPase activity.

In apparent contradiction with these findings, a recent report [3] indicates that under certain conditions calcium uptake by sarcoplasmic reticulum vesicles is not abolished, but is rather increased by ether. Since important information on the reactions of sarcoplasmic reticulum ATPase with nucleotides

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Abbreviation: Mops, 3-(*N*-morpholino)propanesulfonic acid.

and orthophosphate [1,2,4] was obtained with vesicles which were assumed to be unable to accumulate Ca^{2+} as a consequence of ether treatment, it is necessary to clarify the experimental procedures which are required to produce this effect.

Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by homogenization and differential centrifugation as previously described [5]. Protein was determined by the Folin method, standardized with bovine serum albumin.

Treatment with diethyl ether was carried out as originally described [1], by mixing a sarcoplasmic reticulum suspension (0.2–1.0 mg/ml protein in 20 mM Mops buffer (pH 6.8) and 80 mM KCl) with diethyl ether to a concentration of 5–7% (v/v), centrifuging at 40 000 × *g* for 60 min, and resuspending the sedimented vesicles with a hand homogenizer. These suspensions were then used for structural and functional studies. Alternatively, 5–7% diethyl ether (v/v) was added directly to reaction mixtures containing sarcoplasmic reticulum vesicles, and structural-functional studies were performed directly on these suspensions as described by Salama and Scarpa [3].

ATP-dependent calcium uptake by sarcoplasmic reticulum vesicles was measured by monitoring light absorption changes undergone by metallochromic indicators such as murexide [6]. In other experiments, the sarcoplasmic reticulum vesicles were loaded with ^{45}Ca in the presence of oxalate, and then centrifuged in a sucrose gradient in order to measure the radioactive calcium remaining in the supernatant and that accumulated by the vesicles.

ATPase activity was estimated by determination of P_i release, according to the vanadate method developed by LeCocq and Inesi [7], and modified by Lin and Morales [8].

Transient state enzyme phosphorylation and P_i release were studied in rapid quench experiments [9], using radioactively labeled [γ-^{32}P]ATP as the substrate.

Electron microscopy

Suspensions of sarcoplasmic reticulum vesicles were mixed with an equal volume of 5% glutaraldehyde and 2% tannic acid in 0.1 M sodium cacodylate (pH 7.0). These mixtures were allowed to stand 15 min at room temperature, then were centrifuged at 40 000 × *g* for 1 h at 0°C. The resulting pellets were cut into cubes < 1 mm^3, rinsed in 0.014 M veronal-acetate buffer (pH 7.0) containing 2.4 mM $CaCl_2$ [10], and postfixed in 1% osmium tetroxide in the same buffer for 1 h. The samples were then rinsed in the same buffer set to pH 6.0, and fixed again in 0.5% uranyl acetate in that buffer for 1 h. They were then dehydrated in ethanol and proplylene oxide, and embedded in Epon-Araldite resin. Ultra thin silver or grey sections were prepared with a diamond knife, mounted on uncoated 300 mesh copper grids, and stained with 25% methanolic uranyl acetate followed by Reynolds' lead citrate.

Samples for freeze fracturing were prepared by adding 3 ml glycerol to 12 ml (6–7 mg protein) of suspended sarcoplasmic reticulum vesicles. These were incubated for 1 h at 0°C, and then centrifuged at 40 000 × *g* for 1 h at 0°C. Samples of the pellet were frozen in liquid Freon 22, fractured at −100°C, and platinum carbon replicas were made on a Balzers 360 M. The replicas were cleaned for 1 h in bleach, washed, and mounted on uncoated copper grids. Electron microscopic observations were made with a Philips EM 200.

Results

Ultrastructural observations

As first reported by Saito et al. [10], the electron microscopic appearance of sarcoplasmic reticulum vesicles can be improved by addition of tannic acid to the aldehyde fixative and subsequent treatment with uranyl acetate in a modified veronal-acetate buffer after postfixation with osmium tetroxide. Thin sections of these preparations display a heavy coat of darkly stained material on the outer ('cytoplasmic') surface of the vesicles (Fig. 1a). This fuzzy coat corresponds to the outer granules visualized on negatively stained preparations (Fig. 1a, top inset), which are attributed to the polar portions of the amphiphilic polypeptide chains constituting the Ca^{2+}-dependent ATPase [11–14]. These structures are clearly resolved as individual 'lollipop' shaped units in favorable views. Furthermore, very thin

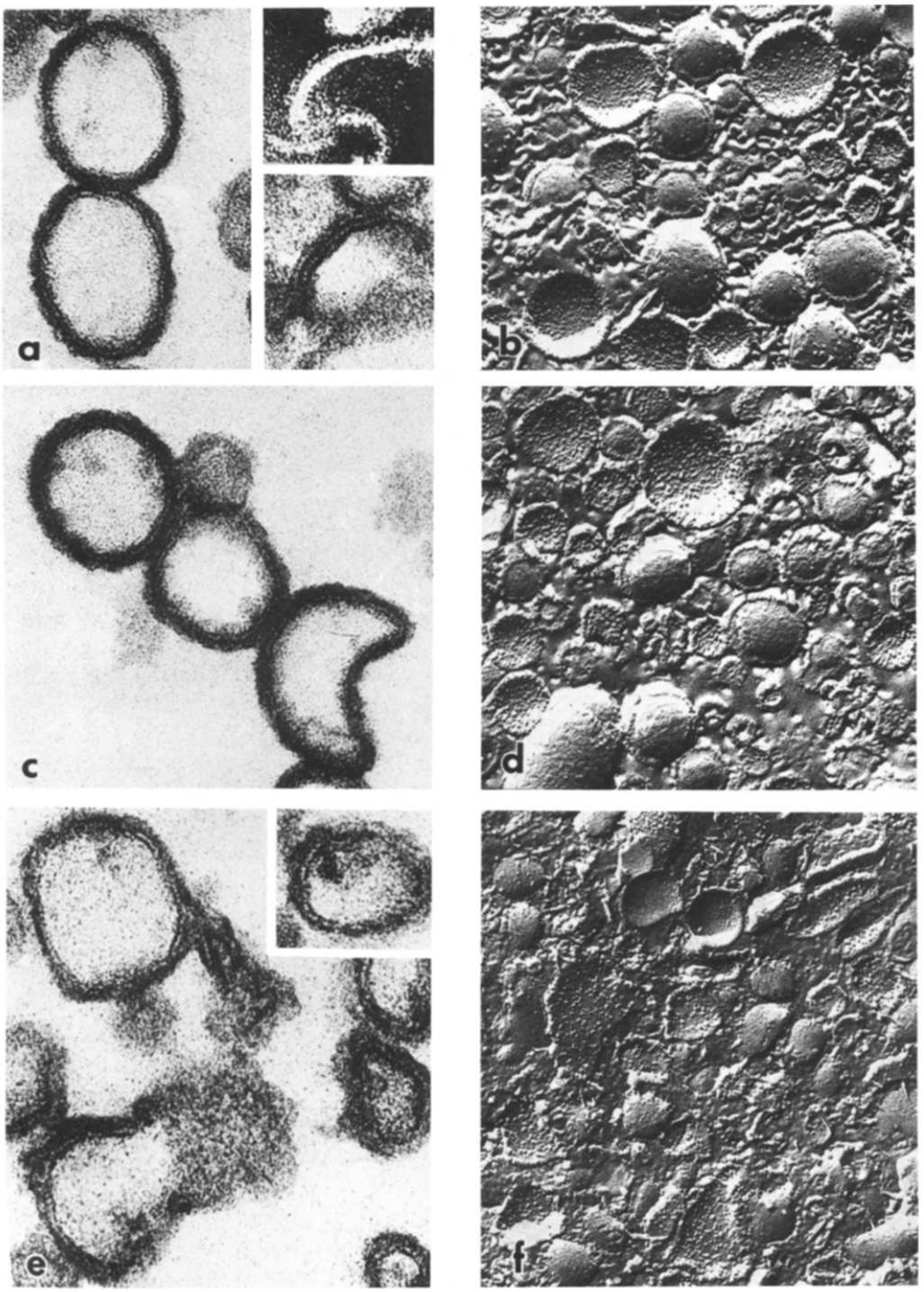


Fig. 1. Electron micrographs of sarcoplasmic reticulum vesicles, before and following treatment with diethyl ether (7%, v/v). (a) Control (untreated) vesicles. Most are round or ovoid with no detectable contents, and after fixation with tannic acid carry a coat of darkly stained material on their outer 'cytoplasmic' surfaces. This fuzzy coat corresponds to the Ca-ATPase polypeptides, which are better resolved as 'lollipop' shaped unit by negative staining (inset, top) or in favorable views of thin sections (inset, bottom) where regularly spaced densities span the bilayer and likely correspond to the lipophilic portions of the ATPase

sections of tannic acid fixed preparations yield exceptionally interesting detail, in that regularly spaced densities can also be seen spanning the bilayer (Fig. 1a, bottom inset); these densities could correspond to the lipophilic portions of the amphiphilic ATPase chains.

The ultrastructural effects of diethyl ether upon sarcoplasmic reticulum vesicles varied with the method of application. No alterations were detected (Fig. 1c) in vesicles which were simply exposed to diethyl ether prior to fixation for times varied from 1 to 15 min. However, vesicles which were exposed to diethyl ether and then were in addition centrifuged and resuspended by hand homogenization before being fixed, displayed a marked transformation (Fig. 1e). In this case, the outer fuzzy coat was markedly discontinuous and the vesicles were aspherical, often displaying bizarre shapes (Fig. 1e, inset). Some fragmentation and clumping of vesicles was also noted.

In agreement with results from thin sections, replicas of freeze-fractured preparations revealed that simple exposure of the sarcoplasmic reticulum vesicles to diethyl ether produced no detectable alterations (Fig. 1, b and d). The vesicles were roughly circular in profile and displayed the distinct asymmetric disposition of intramembranous particles which characterizes sarcoplasmic reticulum membranes either isolated or in intact muscle. The concave or 'P' fracture faces were populated with numerous 9-nm particles attributed to the lipophilic portions of the ATPase chains [15,16], and the convex or 'E' fracture faces were nearly devoid of particles.

While vesicles which were simply exposed to ether did not display any detectable ultrastructural changes, treatment of vesicles with diethyl ether followed by centrifugation and resuspension produced dramatic alterations (Fig. 1f). In this case, the vesicle profiles were irregular and often indistinct. The concave fracture faces had a reduced density of particles or were totally smooth. On the other hand the convex fracture faces, which are normally smooth, acquired a significant number of particles as a consequence of this treatment. In addition, unidentified debris was present in the ice matrix between the vesicles.

Functional observations

Addition of ATP to media containing sarcoplasmic reticulum vesicles, Ca^{2+} and appropriate cofactors, is followed by rapid transport activity producing calcium filling of the vesicles in approx. 60 s. At this time the transport activity is inhibited by the high concentrations of Ca^{2+} accumulated inside the vesicles [17]. Under these conditions, transport activity can be followed by monitoring light absorption changes undergone by metallochromic indicators. It is shown in Fig. 2 that when diethyl ether (5–7%, v/v) is simply added to the reaction mixture as described by Salama and Scarpa [3], both the velocity and maximal levels of calcium uptake are increased (middle trace), as compared to control vesicles (upper trace). On the other hand, if the sarcoplasmic reticulum vesicles are first exposed to an identical concentration of ether but are centrifuged and resuspended before being placed in the reaction mixture, no net calcium uptake is noted upon addition of ATP (lower trace). A reduction of calcium uptake is also observed when the reaction medium containing sarcoplasmic reticulum vesicles and diethyl ether is subjected to homogenization in a tightly fitting hand homogenizer. This maneuver does not produce any effect in the absence of ether.

molecules. (Magnified X194 000; insets magnified X249 000.) (b) Freeze-fracture replica of control vesicles. Most concave faces demonstrate numerous 9-nm diameter particles while convex faces are smooth. (Magnified X75 000.) (c) Vesicles treated with diethyl ether then fixed without further treatment. These are indistinguishable in appearance from the controls, with their fuzzy ATPase coat still intact. (Magnified X194 000.) (d) Fracture faces of vesicles exposed to diethyl ether retain the asymmetrical particle disposition of the control membranes. (Magnified X75 000.) (e) Vesicles treated with diethyl ether, but centrifuged and resuspended before being fixed. These vesicles are markedly different from the control vesicles: the fuzzy ATPase coat is noticeably lacking, and the vesicles are irregular rather than round or ovoid in shape. Some vesicles (inset) have prominent darkly-stained contents which probably are Ca-ATPase molecules aggregated in the vesicle lumen. (Magnified X194 000; inset magnified X225 000.) (f) Fracture faces of vesicles exposed to diethyl ether followed by centrifugation and resuspension. Intramembranous particles are present on both fracture faces. Concave faces are depleted of particles and some are totally empty. Irregularly shaped profiles are common and debris is present in the ice matrix. (Magnified X75 000.)

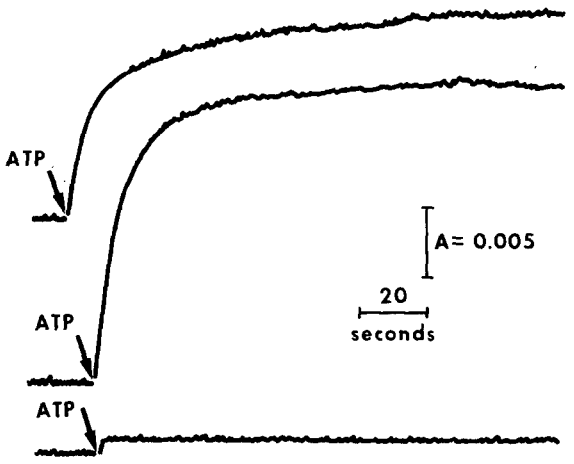


Fig. 2. ATP-dependent Ca^{2+} uptake by sarcoplasmic reticulum vesicles. Upper trace, control vesicle; intermediate trace, in the presence of diethyl ether (7%, v/v) in the reaction mixture; lower trace, vesicles exposed to diethyl ether (7%, v/v), centrifuged and resuspended before being added to the reaction mixture. Ca^{2+} uptake was measured in the presence of 20 mM Mops (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, 50 μM $CaCl_2$, 100 μM murexide and 0.55 mg sarcoplasmic reticulum protein/ml. ATP was added to give a 0.5 mM final concentration to start the reaction. Disappearance of Ca^{2+} from the medium (e.g., Ca^{2+} uptake by the vesicles) was followed by monitoring light absorption changes undergone by murexide in a double wavelength (508 vs. 545 nm) spectrophotometer. Ca^{2+} standardization was identical in the presence and in the absence of diethyl ether. Maximal uptake was 90 and 140 nmol calcium/mg protein for the upper and the intermediate trace, respectively.

It is well known that if ATP-dependent calcium transport by sarcoplasmic reticulum vesicles is permitted to proceed in the presence of oxalate, complexation of the high intravesicular Ca^{2+} by oxalate prevents inhibition of transport activity [18]. As a consequence calcium uptake continues considerably longer, leading to accumulation of large amounts of calcium and to calcium oxalate precipitation inside the vesicles. Owing to their high density the loaded vesicles can be easily centrifuged through a sucrose step gradient and their content of calcium assessed by determinations of radioactive calcium isotope and protein in the pellet. From such experiments we again found that no calcium loading is obtained with vesicles treated with diethyl ether, centrifuged and resuspended by hand homogenization before being added to the reaction mixture for calcium uptake (Table I). In contrast, the ability of the vesicles to accumulate calcium was retained if diethyl ether was simply added to the reaction mixture for calcium uptake. In this case, however, the yield of loaded vesicles upon centrifugation on the sucrose step gradient was reduced, probably due to fragility acquired by the sarcoplasmic reticulum membrane in the presence of diethyl ether and subsequent rupture of loaded vesicles during centrifugation.

TABLE I

CALCIUM LOADING OF SARCOPLASMIC RETICULUM VESICLES IN THE PRESENCE OF OXALATE

C, control vesicles; E, 5% (v/v) diethyl ether present in the reaction mixture; ECH, vesicles exposed to diethyl ether (5%, v/v), centrifuged and then resuspended before being added to the reaction mixture. Loading mixture: 20 mM Mops (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, 2.0 mM EGTA, 2.0 mM EGTA, 2.0 mM $^{45}CaCl_2$, 2–5 mM oxalate, 0.1 mg sarcoplasmic reticulum protein/ml and 10 mM ATP. Following 10 min incubation at 37°C, the reaction mixture was cooled in ice and 7 ml of it were placed over 5 ml 52% sucrose in loading medium. Following centrifugation for 45 min at 82 500 × g in a SW41 Beckman rotor, the pellets were recovered for determination of protein and radioactive calcium. Data in the table refer to the percentage of sarcoplasmic reticulum protein recovered in the pellet relative to the amount placed on top of the step sucrose gradient. Calcium loading is referred to the protein determined in the pellet.

Sample	Loaded vesicles (% yield)	Loading (μmol calcium/ mg protein)
C	47 –55	12–20
E	15 –45	11–23
ECH	0.1– 0.5	5–12

Utilization of ATP for calcium transport entails hydrophobic cleavage of the terminal phosphate of ATP, catalyzed by the Ca^{2+}-dependent ATPase by a mechanism known to include a phosphorylated enzyme intermediate. Ca^{2+}-dependent hydrolytic activity can be easily measured in the presence of oxalate, since in these conditions transport activity is permitted to proceed at high rates for a few minutes, after which transport and hydrolytic activity decline simultaneously when maximal calcium loading of the vesicles is attained [18]. A similar pattern (Fig. 3) was obtained when diethyl

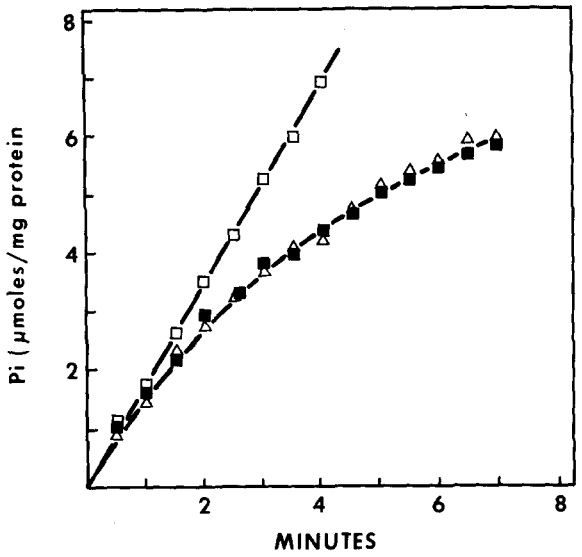
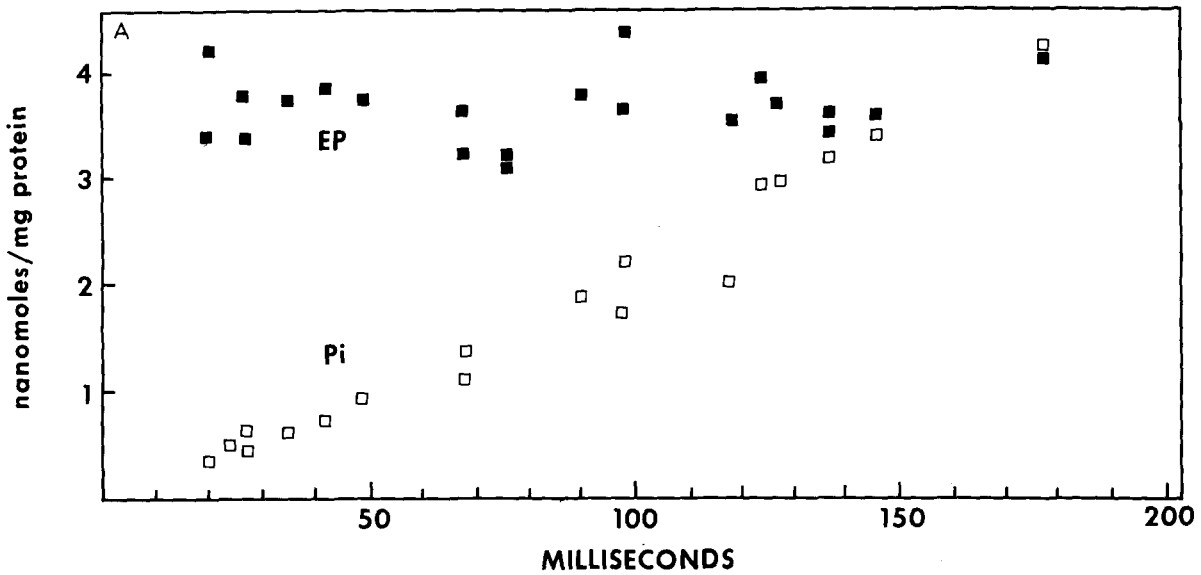


Fig. 3. Ca^{2+}-dependent ATPase activity in the presence of oxalate. ■, control vesicles; △, in the presence of diethyl ether (7%, v/v) in the reaction mixture; □, vesicles treated with diethyl ether (7%, v/v), centrifuged and resuspended before being added to the reaction mixture. ATPase activity was measured in the presence of 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.9 mM $CaCl_2$, 2 mM oxalate, 0.05 mg sarcoplasmic reticulum protein/ml and 2 mM ATP. The reaction was stopped at serial times by addition of the ammonium molybdate reagent (Lin and Morales [8]). Ca^{2+}-independent ATPase was subtracted from the data shown in the figure.



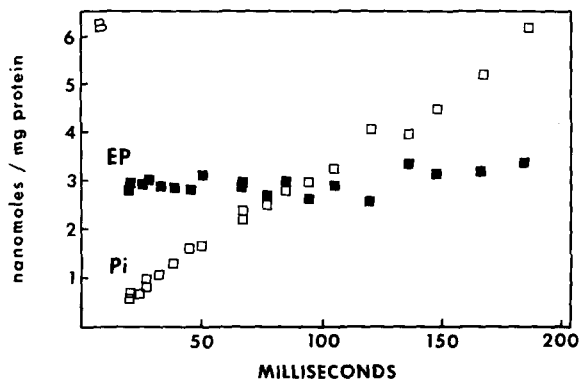


Fig. 4. Phosphoenzyme (EP) and P_i production following addition of ATP to sarcoplasmic reticulum vesicles. (A) Control vesicles. (B) Presence of diethyl ether (7%, v/v) in the reaction mixture. The reaction was started in a quench flow apparatus. In all experiments: Syringe A: 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$ and 0.2 mg sarcoplasmic reticulum protein/ml, and 7% diethyl ether when indicated; Syringe B: same as in syringe A, except for 40 μM [γ-^{32}P]ATP instead of sarcoplasmic reticulum vesicles; Syringe C: 0.25 M perchloric acid and 4 mM unlabeled P_i.

ether was simply added to the reaction mixture, indicating that under these conditions the vesicles retained their ability to accumulate calcium (Fig. 2 and Table I). However, if after being treated with diethyl ether the vesicles were centrifuged and resuspended prior to being added to the reaction mixture, the Ca^{2+}-dependent ATPase activity did not undergo the usual time-dependent decline, but continued at constant rates (Fig. 3) until the substrate was depleted. This pattern is consistent with the inability of these vesicles to accumulate calcium (Fig. 2 and Table I).

Our observations described so far demonstrate that diethyl ether produces obvious structural changes and impairs the ability of sarcoplasmic reticulum vesicles to accumulate calcium, if the vesicles are centrifuged down and resuspended following exposure to diethyl ether, according to the procedures used by Inesi et al. [1], and Fiehn and Hasselbach [2]. On the other hand, if the vesicles are simply exposed to ether added to the reaction mixture as reported by Salama and Scarpa [3], obvious structural changes are not produced, and the ability of the vesicles to accumulate calcium is not impaired. In fact, a stimulation of calcium transport activity is observed when calcium transport is measured in the absence of oxalate (Fig. 2).

To further characterize the phenomenon of stimulation of calcium transport activity by diethyl ether, we measured phosphoenzyme formation and P_i production in the early part of the reaction following addition of ATP. We found (Fig. 4) that while the phosphoenzyme levels were not increased in the presence of diethyl ether, P_i production was significantly higher, indicating a faster phosphoenzyme turnover. This suggests that the presence of ether affects intermediate reactions which precede the step of phosphoenzyme hydrolytic cleavage and are normally rate limiting in the transport and ATPase cycle.

It should be pointed out that passage of the reaction mixture containing vesicles, murexide and diethyl ether (8%, v/v) through the rapid mixing chamber of the Durrum apparatus does not damage the vesicles. This was established in experiments in which the reaction mixture was passed through the mixer in the absence of ATP, and then tested for calcium uptake following addition of ATP in the cuvette of a double wavelength spectrophotometer. It was found that the brief (milliseconds) flow through the mixer did not produce shearing of the vesicles as demonstrated by retention of calcium uptake activity and lack of penetration of murexide inside the vesicles. On the other hand, the shearing produced by hand homogenization can be shown by penetration of murexide inside the vesicles.

Discussion

Our studies indicate that diethyl ether confers to the sarcoplasmic reticulum membrane a state of lability whereby sarcoplasmic reticulum vesicles undergo pronounced structural alterations when subjected to mechanical perturbations which in the absence of diethyl ether have no effect on the sarcoplasmic reticulum vesicle structure. The alterations include a striking randomization of the 9-nm diameter intramembranous particles to both the concave and convex membrane faces, from their distribution almost exclusively on the concave faces which is typical of normal sarcoplasmic reticulum vesicles [14]. Thus the asymmetric character of Ca-ATPase distribution in the sarcoplasmic reticulum membrane is lost. It should be pointed out that a similar randomization of ATPase particles occurs when the amphiphilic character of the ATPase units is altered by prolonged digestion with trypsin [14].

Not surprisingly, vesicles undergoing such pronounced structural alterations lose their ability to accumulate calcium in the presence of ATP, although ATPase activity is sustained at normal levels. This effect may be due to increased permeability of the vesicle membranes produced by the diethyl ether treatment [19]. It is interesting that vesicles exposed to diethyl ether without being subjected to centrifugation and resuspension do not lose but actually display an improved ability to transport calcium. This effect is not produced by recruitment of a greater number of ATPase pump units; rather, a higher turnover rate of nearly unchanged amounts of phosphorylated enzyme intermediate is demonstrated in rapid quench experiments (Fig. 4). In addition to a faster enzyme turnover, the final levels of calcium accumulated in the absence of oxalate are also increased. This suggests that exposure to diethyl ether loosens the packing of

the membrane lipids and facilitates adjustment of the vesicles to osmotic gradients which are generated by Ca^{2+} transport.

In contrast to the enhancing effects of diethyl ether on sarcoplasmic reticulum vesicles with respect to calcium uptake velocity and maximal loading levels as well as on turnover of the phosphoenzyme intermediate, centrifugation and resuspension of diethyl ether treated vesicles abolishes these capabilities, probably by increasing the permeability of the vesicles [2]. Concomitantly, striking ultrastructural alterations are observed which are totally consistent with increased fragility and with gross displacement of Ca-ATPase pump units with respect to the membrane bilayer of the sarcoplasmic reticulum vesicle. The presence of cholesterol in membranes is known to affect the packing of lipids and the fluidity of the membrane bilayer (for review, see Ref. 20). Replacement of membrane cholesterol even by closely-related steroid increases both the permeability and fragility of erythrocytes [19], and it has been reported that at the concentrations used in the study, diethyl ether selectively extracts cholesterol esters from sarcoplasmic reticulum vesicles [20]. It is apparent that ether weakens lipid and protein interactions to a degree facilitating specific protein motions involved in the transport and catalytic cycle. Furthermore, it is likely that this effect permits protein displacement and randomization following mechanical perturbations that normally do not produce irreversible alterations.

This report on the loss of the asymmetrical disposition of Ca-ATPase pump units may be of considerable relevance for studies requiring centrifugatins and homogenizations in the presence of lipotropic agents such as organic solvents and detergents.

Acknowledgements

We are grateful to Susan Keating for technical assistance. This research was partially supported by Grants of the NIH (HL 16607 and Biomedical Research Support 5S07 RR05301), the Muscular Dystrophy Association and the Pacific Research Foundation.

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