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MODE OF ACTION OF DIETHYL ETHER ON ATP-DEPENDENT Ca^{2+} TRANSPORT BY SARCOPLASMIC RETICULUM VESICLES

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Abstract—The mode of action of diethyl ether on the sarcoplasmic reticulum Ca^{2+} pump and ATPase activity was investigated in sarcoplasmic reticulum vesicles (SR). The addition of ether (5%, v/v) at 23° nearly doubled the rates of ATP-dependent Ca^{2+} uptake and ATP hydrolysis by SR for a wide range of ATP concentrations (20 μM to 10 mM). By contrast, the Ca^{2+}-independent ATPase acivity of SR decreased with increasing ether concentrations and practically ceased at higher ether solutions (5–7%, v/v). Ether not only enhanced the forward transport of Ca^{2+} into vesicles but also the reversal of the Ca^{2+} pump, and higher rates of Ca^{2+} efflux coupled to ATP synthesis were observed from Ca^{2+}-loaded vesicles. Electron micrographs of SR pellets showed that the average radius of the vesicles increased by about 20% upon exposure to ether. In the range of 5–40° the rate of Ca^{2+} transport increased with temperature; at about 4° active Ca^{2+} transport by SR normally ceased, but with the addition of ether (5%, v/v) significant Ca^{2+} transport (1–2 nmoles Ca^{2+} per mg per sec) occurred at 0°. The further stimulation of SR Ca^{2+} transport induced by ether was particularly effective at low temperatures; ether increased the rate of Ca^{2+} uptake by a factor of 10 at 5° but only by a factor of 1.5 at 40°. The effect of ether on the (Ca^{2+}–Mg^{2+})ATPase of SR could be reversed by resuspending the vesicles in ether-free media. The centrifugation and resuspension of SR in ether-free solutions did not make the vesicles leaky nor did it lyse them irreversibly unless they were also mechanically disrupted. The results indicate that, in ether, there was an increase of intravesicular volume and an increase in membrane fluidity of SR, which could account for the dramatic increase in Ca^{2+} capacity and rate of Ca^{2+} transport of ether-treated SR.

Pharmacological concentrations of diethyl ether are well-known to depress the time to peak and the peak developed tension of cardiac [1] and skeletal [2] muscle fibers. Various studies on the effect of ether on skeletal muscle at the cellular level have convincingly demonstrated that the metabolism [3], resting potential [4], action potential [4, 5], and contractile response to myofibrils [6] remain essentially unaltered by ether. Consequently, it has been proposed [1, 6] that ether reduces cytosolic Ca^{2+} during the contraction cycle through a pharmacological effect at the level of the sarcoplasmic reticulum. This view, however, has long been contradicted by biochemical studies on isolated sarcoplasmic reticulum vesicles (SR) in which treatment of SR with ether was reported to lyse or make the vesicles leaky and to prevent ATP-dependent Ca^{2+} accumulation [7, 8]. The uncoupling of Ca^{2+} uptake from ATP hydrolysis implied that the action of ether on SR did not account for the relaxation of muscles exposed to ether since SR that are leaky to Ca^{2+} should produce a state of rigor rather than relaxation. Moreover, for several years, ether treatment provided a convenient experimental system to measure steady-state ATPase activity at "known" Ca^{2+} concentrations. The initial bursts of ATPase activity, which normally last a few seconds, were thought to be prolonged by ether for minutes and not to be rapidly inhibited by high Ca^{2+} concentrations sequestered inside the vesicles. Instead, the rate of ATPase activity was related to the ATP and calcium concentrations in the reaction medium, since Ca^{2+} in the intra- and extravesicular medium remained in equilibrium. Moreover, ether treatment was reported not to affect the "basal" of Ca^{2+}-independent ATPase activity, whereas other agents available to lyse vesicles (i.e. Triton X-100) significantly altered basal and/or active rates of ATP hydrolysis by SR [7, 9].

In contrast, more recent experiments have shown that ether does not lyse or render SR leaky to Ca^{2+} but increases both rates of Ca^{2+} transport and ATP hydrolysis by a factor of 2 and increases the total Ca^{2+} accumulated by the vesicles by about 80% [10, 11]. These contradictory findings may have arisen from the different experimental procedures used to administer ether. In earlier experiments, the SR were "ether-treated", which meant that they were suspended in ether solutions, centrifuged, and then resuspended in ether-free medium, whereas in more recent studies ether was simply added to the SR reaction mixture.

In the present experiments, "ether treatment" tantamount to washing the SR in ether-free medium after exposure to ether did not make them leaky but simply reversed the ether-induced stimulation of

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Ca^{2+} transport by SR back to normal values. The purpose of this study was then to investigate the mode of action of ether on ATP-dependent Ca^{2+} transport by SR so that the pharmacologic effect of ether on muscle function could be better understood on a biochemical level.

EXPERIMENTAL PROCEDURES

Preparation of SR. Sarcoplasmic reticulum vesicles were prepared from white skeletal muscle of rabbit hind leg as described previously [12]. After the last centrifugation step, the isolated SR were suspended in a medium consisting of 0.9 M sucrose, 10 mM histidine (pH 6.8) at concentrations of 8–12 mg protein/ml and were stored in liquid nitrogen until use. Protein concentrations were determined by the method of Lowry *et al.* [13].

Purification of SR. In some experiments, SR were further purified with a sucrose step gradient to measure Ca^{2+}-dependent and Ca^{2+}-independent ATPase activities in the absence of possible mitochondrial or other organelle contaminants. The following sucrose solutions (% by weight) plus 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) at pH 7.0 were layered sequentially in centrifuge tubes: 4 ml of 37.2%, 7 ml of 33.9%, 3 ml of 31.6%, 8 ml of 29.1%, 2 ml of 26.4% sucrose, and finally 2 ml of a double-concentrated SR suspension (~20 mg protein/ml). The densities of the sucrose solutions were checked with a refractometer (Bausch & Lomb, Rochester, NY), and the tubes were centrifuged at 22,000 rpm in an SW 27 rotor for 12 hr (Beckman, Analytical Ultracentrifuge L-2B, Fullerton, CA) at 4°. Fractions from 30–32.5% sucrose, 33.5–38% sucrose, and 38.5–42% sucrose contained the purified light, intermediate, and heavy SR fractions respectively [14]. The three bands were combined, diluted by a factor of 10 in 100 mM KCl, 10 mM $MgCl_2$, 2 mM Tris–maleate, centrifuged at 18,500 rpm in an SS-34 Sorvall rotor (Dupont Instruments, Sorvall Operations, Newtown, CT) for 90 min at 4°, and resuspended at a concentration of 6–9 mg SR protein/ml of KCl reaction medium for subsequent assays of inorganic phosphate.

Addition of diethyl ether to SR suspensions. At the start of an experimental run, concentrated SR suspensions kept on ice were diluted to 0.2 to 2.0 mg protein/ml in 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris–maleate, pH 6.9, at 23° (unless otherwise stated). Diethyl ether was then added to the reaction mixture as a percentage of final volume and immediately mixed into the aqueous medium by covering the vial (or cuvette) and inverting it upside-down five times. Most measurements of Ca^{2+} uptake and/or ATP hydrolysis lasted about 10 min, during which time the vials were kept open and the surface of the SR reaction mixtures was exposed to room air.

"Ether-treated" SR. Ether treatment of SR was achieved by first adding various concentrations of diethyl ether (1–5%, v/v) to an SR suspension and mixing the ether into solution by inverting the tubes upside-down five times. The SR suspended in ether-containing solutions were then centrifuged for 90 min at 18,500 rpm in an SS-34 Sorvall rotor, and the pellets were resuspended in ether-free medium with a glass homogenizer with a 0.13–0.18 mm clearance (Arthur H. Thomas, Philadelphia, PA) by manually pushing the teflon pestle five to ten times in and out of the barrel. The protein concentrations of "ether-treated" SR were re-measured by the method of Lowry *et al.* and were found to be comparable to values prior to ether treatment.

Electron microscopy. The morphological alterations induced by ether were re-examined by electron microscopy using tannic acid in the fixative to improve the appearance of the vesicles and the resolution of the SR membrane as described by Saito *et al.* [15]. Vesicles were suspended in 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris–maleate, 200 μM $CaCl_2$ at pH 6.9, concentrated to 25 mg protein/ml, and sedimented in the presence or absence of 5% diethyl ether in a 150-μl tube with a Beckman Airfuge at 98,000 *g* for 2 min. Pellets were fixed for 2 hr at 0° in 3% gluteraldehyde, 0.1 M sodium cacodylate (pH 7.2) containing 1% tannic acid as previously described [15]. Samples were stored in 0.1 M sodium cacodylate, then post-fixed in 1% OsO_4 for 1–2 hr, dehydrated in ethanol, stained with 1% uranyl acetate in 95% ethanol, and embedded in epon. They were sectioned perpendicularly to the surface of the pellets and stained with uranyl acetate and lead. Pictures were taken with an AE1 100B microscope and were analyzed with an OMNICON automatic image scanner (Bausch & Lomb) to measure changes in the average diameters of the vesicles. Electron micrographs were placed in the image field of the instrument, which was programmed to search for all topographically closed particles (0.04 μm or greater), calculate their average diameter, plot a histogram of their size distribution, and determine the mean diameter of the vesicle population sampled by the image analyzer.

Measurements of Ca^{2+} uptake and ATP hydrolysis. Ca^{2+} uptake was measured spectrophotometrically using arsenazo III as an indicator of extravesicular Ca^{2+} concentration in the suspending medium, and its differential absorbance was measured kinetically with a time-sharing dual wavelength spectrophotometer at 685–675 nm [16]. The use of arsenazo III, its purification, and the standard controls that demonstrate its specificity for Ca^{2+} in similar experiments have been reported previously [17]. The presence of diethyl ether did not affect the absorption characteristics of arsenazo III nor did it hinder its ability to monitor specifically and quantitatively the changes of Ca^{2+} concentration. Simultaneous kinetic measurements of Ca^{2+} uptake and ATPase activities were carried out in the same magnetically stirred cuvette by recording the Ca^{2+} uptake through the absorption changes of arsenazo III and the acidification of the medium due to ATP hydrolysis with a small diameter, fast-response pH combination electrode (A. H. Thomas; 4094-L60). Under our experimental conditions (pH 7.0), in the absence of SR, 1.0 mole of H^+ was released per mole of ATP hydrolyzed, in agreement with the results of Herbette *et al.* [18] who used a similar experimental protocol.

As an alternative, the ATPase activity was also determined by measuring the appearance of inor-

ganic phosphate (P_i) in the reaction mixture. Either 200 μM Ca^{2+} or 200 μM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) was added to the medium to measure Ca^{2+}-dependent or Ca^{2+}-independent ATPase activities respectively. The release of P_i was measured upon the addition of ATP (2 mM) to magnetically stirred SR reaction mixtures (10 ml). Aliquots of 500 μl were withdrawn at regular time intervals into an equal volume of ice-cold ammonium molybdate-vanadate reagent and trichloroacetic acid to interrupt the reaction, as previously described by Lecoq and Inesi [19]. The samples were centrifuged to remove precipitated proteins, and P_i concentrations in the supernatant fractions were measured through the absorbance of the phosphomolybdo–vanadate complex at 400 nm. The presence of up to 10% (v/v) ether in the SR reaction mixtures did not perturb the extraction or the colorimetric assay of P_i.

Concentration of ether in the medium as a function of time. In several control experiments, SR were incubated in ether solution for 20–30 min in either sealed or open vials before measurements of ATP-dependent (active) Ca^{2+} transport. Even though ether is highly volatile, such prolonged incubations in either open or shut vials did not alter significantly the effect of the anesthetic on active Ca^{2+} transport.

Nevertheless, the extent of ether evaporation from the aqueous phase was determined by measurements of ether concentration as a function of time using a Varian 3700 gas chromatograph (Varian Instruments Division, Palo Alto, CA). Diethyl ether (5 or 10%, v/v) was mixed into 2 ml of medium with or without SR (1 mg/ml) in a standard (1 × 1 cm) cuvette which was kept open at 23°. Aliquots (10 μl) taken at chosen time points were injected quickly into sealed tubes containing CCl_4 (1 ml) to extract ether from the aqueous phase and chloroform (10 μl), a reference solute for chromatography. Sealed tubes with samples taken at various times were vortexed and centrifuged (10 min × 5000 rpm). An aliquot of 10 μl from each tube was injected in the gas chromatograph to measure the levels of ether (in mg ether/ml reaction mixture) at each time point. These measurements were highly accurate and reproducible since the extraction of ether by CCl_4 is nearly 100% efficient, and experimental variations were within ±1%.

Concentration of diethyl ether in the lipid phase of the vesicles. The diethyl ether concentration in the lipid bilayer of SR and its partition coefficient between the lipid and aqueous phases were determined by gas chromatography. SR (7.2 mg protein) were suspended in 10 ml of 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–maleate, pH 7.0, at 5° with either 1.25, 2.5, 5.0, or 10% diethyl ether. Tubes were kept sealed and were centrifuged for 90 min × 45,000 *g* to obtain tight SR pellets. Aliquots (10 μl) of the supernatant fractions were injected in sealed test tubes containing 1 ml CCl_4 + 0.02% chloroform. The test tubes were vortexed and centrifuged, and the ether extracted from each sample of supernatant fraction was measured with the Varian 3700 gas chromatograph. Supernatant fractions were discarded, the solution around each pellet was carefully aspirated, and 4 ml of CCl_4 + 0.02% chloroform was added to each pellet. The pellets in CCl_4 were vortexed and centrifuged, and the ether content of each pellet was measured by gas chromatography. In parallel experiments under identical conditions, the total aqueous space in the pellets was determined by measuring the water volume of ^3H_2O [10, 11]. The ether concentration in the lipid phase was obtained from the total ether content in the pellet minus the ether in the water space of the pellets; the later value was calculated by multiplying the water volume in the pellet by the ether concentration measured in the supernatant fraction above the pellet.

Reversal of the Ca^{2+} *pump.* The synthesis of ATP that was coupled with the efflux of Ca^{2+} from Ca^{2+}-loaded SR was measured in the presence and absence of diethyl ether using an experimental protocol similar to that described by Makinose [20]. SR (0.03 mg protein/ml) were Ca^{2+} loaded in an 80-ml medium containing (in mM): KCl, 100; Tris–maleate, 20; $MgSO_4$, 5; $CaCl_2$, 1; EGTA, 1; ATP, 2; and P_i, 20 at pH 6.5, 23° for 3–5 min. The Ca^{2+}-loaded vesicles were centrifuged at 18,500 rpm in an SS-34 Sorvall rotor for 90 min and resuspended at about 1 mg protein/ml in an efflux medium (in mmoles/l) containing: KCl, 100; Tris–maleate, 20; $MgSO_4$, 10; Tris–EGTA, 5; glucose 10; and hexokinase (0.02 mg/ml). The formation of [γ-^{32}P]ATP was initiated by diluting 1 : 1 the Ca^{2+}-loaded vesicles in a similar efflux solution containing 20 mM ($^{32}P_i$ + P_i) plus 2 mM ADP. Aliquots (0.3 ml) of this reaction mixture were withdrawn at regular time intervals into centrifuge tubes containing 0.7 ml of ice-cold perchloric acid (10%) and 5 mM ATP to stop the reaction. The tubes were spun to remove precipitated protein, and newly synthetized [γ-^{32}P]ATP was measured as [^{32}P]glucose-6-phosphate after its extraction from $^{32}P_i$ with 0.4 ml molybdate, 0.2 ml acetone, and 2 ml buffer. The extraction of P_i was repeated three times, and [^{32}P]glucose-6-phosphate was determined by scintillation counting. Control values of [^{32}P]glucose-6-phosphate concentrations were obtained from parallel experiments where the same Ca^{2+}-loaded SR preparation was eluted in efflux media lacking ADP, with or without 5% diethyl ether.

To measure "active" Ca^{2+} efflux, vesicles were loaded in a reaction medium containing 1 mM $^{45}CaCl_2$, centrifuged, and resuspended in efflux medium as described above [21, 22]. "Active" or "passive" $^{45}Ca^{2+}$ efflux was initiated by adding 2 mM ADP + 20 mM P_i or only 20 mM P_i respectively. Aliquots (150 μl) of these reaction mixtures were centrifuged at regular time intervals after the additions of either ADP + P_i or P_i in an ultrafuge (Beckman) for 2 min. The $^{45}Ca^{2+}$ concentration released in the supernatant fraction and the $^{45}Ca^{2+}$ trapped in the SR pellets were measured by scintillation counting under four experimental conditions: with or without diethyl ether and with or without ADP in the efflux medium.

Materials. All reagents were of analytical grade. Anhydrous diethyl either was purchased from the J. T. Baker Chemical Co. (Phillipsburg, NJ); pure grade arsenazo III and NaATP were from the Sigma Chemical Co., (St. Louis, MO); $^{45}CaCl_2$ (4–30 Ci/g of calcium) was from New England Nuclear (Boston,

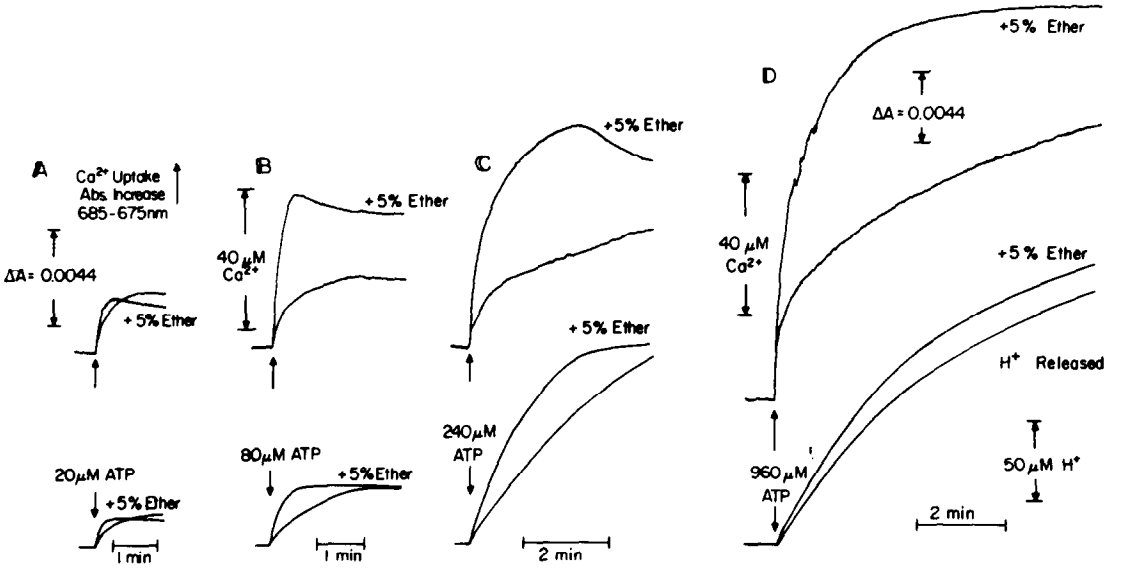

Fig. 1. Ca^{2+} uptake and ATP hydrolysis by SR at various ATP concentrations in the presence or absence of 5% diethyl ether. Ca^{2+} uptake (top traces, A–D) and ATP hydrolysis (bottom traces A–D) were measured simultaneously in a magnetically stirred cuvette through the differential absorption changes of arsenazo III at 685–675 nm and the acidification of the medium with a fast-response pH electrode. The reaction mixtures (2.4 ml) contained 0.85 mg SR protein/ml, 100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris–maleate, 100 μM $CaCl_2$, and 100 μM arsenazo III, at pH 7.0. Either 125 μl of buffer or of diethyl ether was added and mixed in by inverting the covered cuvette five times. The reaction was initiated by additions of Mg-ATP; (A) 20 μM, (B) 80 μM, (C) 240 μM, and (D) 960 μM. Temperature: 23°.

MA), and $^{32}P_i$ (20 mCi/mmole) was from Amersham (Arlington Heights, IL).

RESULTS

Functional changes of SR in the presence of diethyl ether. In Figure 1 (A–D), the effect of diethyl ether (5%, v/v) on Ca^{2+} uptake and ATPase activity was examined over a wide range of ATP concentrations (A: 20 μM, B: 80 μM, C: 240 μM and D: 960 μM). In the presence of 5% diethyl ether, the rates of Ca^{2+} uptake (Fig. 1, top traces) and ATPase activity (Fig. 1, bottom traces) were enhanced for all ATP concentrations (tested up to 10 mM ATP), and the total Ca^{2+} uptake was increased for ATP concentrations greater than 20 μM.

The average rates of Ca^{2+} uptake (Fig. 2A) and ATPase activity (Fig. 2B) during the first 5 sec of the reaction were determined from the slopes of traces similar to those shown in Fig. 1 and were plotted as a function of (ATP). The apparent Michaelis–Menten constants, V_{max} and K_m, were obtained from the y-intercepts and slopes of Lineweaver–Burk plots of (velocity of Ca^{2+} uptake)$^{-1}$ vs (ATP)$^{-1}$ and (velocity of ATP splitting)$^{-1}$ vs (ATP)$^{-1}$ (not shown). An important and well-recognized feature of the SR (Ca^{2+}–Mg^{2+})ATPase is that the velocities of Ca^{2+} uptake and ATPase activity exhibit a biphasic dependence on the substrate concentration [23, 24]. At 0.1 mM ATP, sufficient to saturate the enzyme phosphorylation sites, the rate of transport appeared to reach

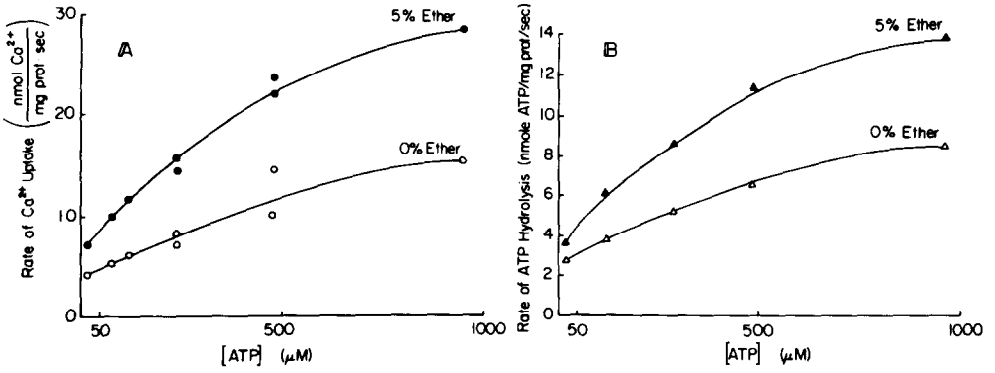

Fig. 2. Rate of Ca^{2+} uptake and ATP hydrolysis by SR in the presence or absence of diethyl ether. From data obtained under experimental conditions described for Fig. 1, the rates of Ca^{2+} uptake (A) and ATP hydrolysis (B) were averaged over the initial 5 sec after the addition of ATP and plotted as a function of ATP concentration.

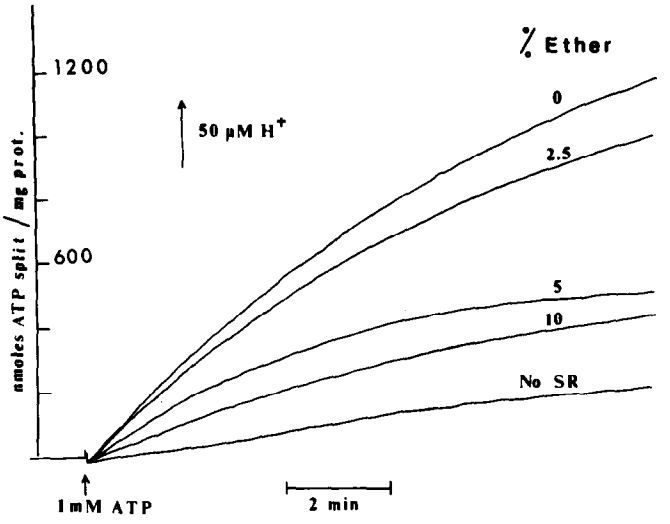

Fig. 3. Ca^{2+}-independent ATPase activity of SR in the presence of various concentrations of diethyl ether. The SR suspension contained (in mM): 100 KCl, 10 $MgCl_2$, 1.0 EGTA, 5 Tris–maleate (pH 7.0), 0.25 mg SR protein/ml, and various amounts of diethyl ether as indicated; the temperature was 23°. The reaction was initiated with the addition of 1 mM ATP, and ATPase activity was measured through the acidification of the reaction mixture.

a maximum value. However, millimolar concentrations of ATP further stimulated the velocity of Ca^{2+} transport and ATPase activity, not through an increase in the levels of phosphoenzyme sites but perhaps through an increased turnover rate of the pump via a positive cooperativity effect of enzyme aggregates [25]. From Lineweaver–Burk plots, the presence of diethyl ether was found to stimulate the velocity of the reaction at low (0.1 mM) and high (1.0 mM) ranges of ATP concentrations. The maximum velocity (V_{max}) of ATP hydrolysis during the first 5 sec of the reaction increased in the presence of ether from 3.6 to 5.7 nmoles ATP hydrolyzed per mg protein per sec for low ATP concentrations and from 8.3 to 15.4 nmoles of ATP hydrolyzed per mg protein per sec for ATP concentrations above 100 μM. Similarly, the maximum velocity (V_{max}) of Ca^{2+} transported by the SR increased in the presence of ether from 5.8 to 10.3 nmoles Ca^{2+} per mg protein per sec for low ATP concentration, and from 13.3 to 29.4 nmoles Ca^{2+} per mg protein per sec for ATP concentrations above 100 μM. Ether increased the K_m value for ATP from 0.72 to 1.2 μM, and from 14 to 20 μM for ATP concentrations below and above 100 μM respectively.

Ca^{2+}-independent and -dependent ATPase activity

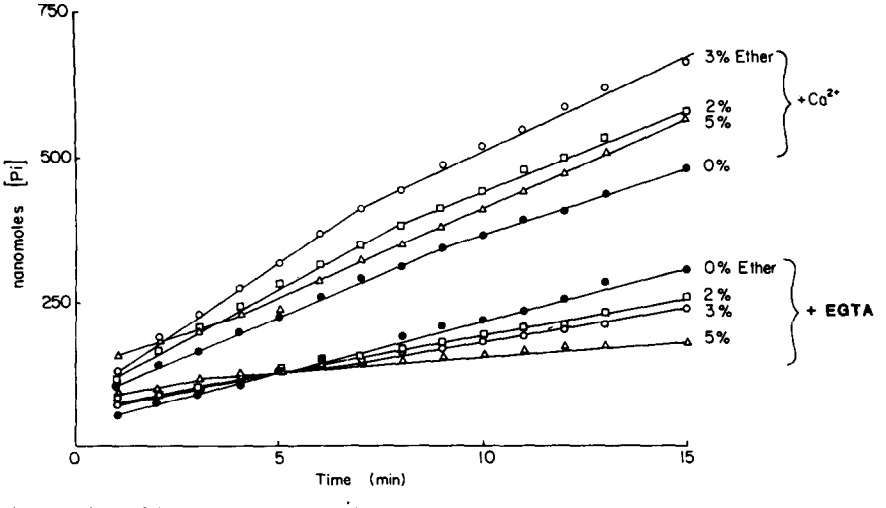

Fig. 4. "Total" and basal ATPase activities of SR suspended in various ether solutions. Purified SR were suspended at a concentration of 0.1 mg protein/ml in media (in mM): 100 KCl, 10 $MgCl_2$, 30 Tris–maleate (pH 7.0), 0.2 mM EGTA (lower traces) or 0.2 mM $CaCl_2$ (upper traces) and either 0% (●), 2% (□), 3% (○), or 5% (△) diethyl ether. For basal (lower traces) and "total" (upper traces) (that is Ca^{2+}-dependent plus basal) ATPase activities, the liberation of P_i was measured, as described in Experimental Procedures, after the addition of 2 mM ATP.

in the presence of ether. The rate of Ca^{2+}-independent (basal) ATP hydrolysis by SR measured through the acidification of the medium (1 mM EGTA) decreased with increasingly greater concentrations of diethyl ether (Fig. 3). In view of the prevailing controversy regarding the source of basal ATPase activity in SR preparations [26] and because of possible contaminations, the rates of basal and active ATPase activities were also measured with purified vesicles through the release of P_i in the medium.

As shown in Fig. 4, the basal ATPase activity measured in a medium containing 200 μM EGTA (lower traces) was inhibited by increasing concentrations of diethyl ether. At 5% ether, the basal ATPase activity virtually ceased since the rate of P_i appearance was similar to control experiments done at room temperature in the absence of SR. With 200 μM Ca^{2+} in the medium, the total rate of ATP hydrolysis (i.e. Ca^{2+}-dependent plus basal activities) was enhanced (Fig. 4, top traces) in the presence of 2 and 3% diethyl ether and levelled off at 5% diethyl ether. Stimulation of the total rate of ATP hydrolysis at 2 and 3% ether was due to increased rates of Ca^{2+}-dependent ATPase activity despite reduced basal rates of hydrolysis. At 5% ether in the medium, the total rate of ATP hydrolysis was lower than at 2 or 3% ether because of a substantial decline in basal activity and levelling off of the ether-induced stimulation of Ca^{2+}-dependent ATP hydrolysis. Thus, the Ca^{2+} ATPase activity was gradually enhanced by increasingly greater concentrations of ether up to 5% (v/v).

Effect of ether on the reversal of the Ca^{2+} pump. Net synthesis of ATP by Ca^{2+}-loaded SR increased monotonically with time producing about 10 nmoles ATP per mg protein per min in the absence of ether and 25 nmoles ATP per mg protein per min in the presence of 5% ether (Fig. 5B). In parallel experiments, the rate of ADP-dependent Ca^{2+} efflux was determined by loading the vesicles with $^{45}Ca^{2+}$ in the presence of ATP and then resuspending them in one of four efflux media containing 20 mM P_i, ± 2 mM ADP, and ±5% diethyl ether. After the addition of P_i, with or without ADP, the SR suspensions were centrifuged after a known delay for a fixed time interval (2 min) with an airfuge. The $^{45}Ca^{2+}$ trapped in the pellets or released in the supernatant fractions was measured by scintillation counting. The total Ca^{2+} released from SR incubated in ADP + P_i minus the "passive" Ca^{2+} efflux from SR suspended in ADP-free media was taken to be the component of net Ca^{2+} efflux. Conversely, $^{45}Ca^{2+}$ trapped in SR pellets preincubated without ADP minus $^{45}Ca^{2+}$ in pellets preincubated in ADP + P_i also represented the net "active" Ca^{2+} efflux. The time resolution of this method is admittedly poor because it requires a minimum of 2 min to spin down the vesicles and thereby stop the reaction by separating the SR from the surrounding medium. Nevertheless, the protocol may be the best, if not the only, alternative to measure the rates of efflux in ether solutions in view of the fact that Millipore filtration did not satisfactorily trap SR in ether solutions (see Fig. 10), and neither metallochromic dyes nor Ca^{2+} electrodes

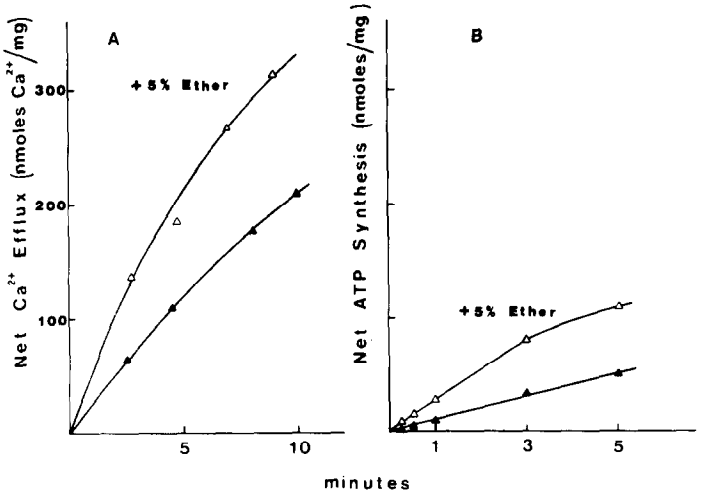

Fig. 5. Net Ca^{2+} efflux (A) and ATP synthesis (B) in the presence (△) or absence (▲) of diethyl ether (5%, v/v) by Ca^{2+}-loaded SR. For measurements of net Ca^{2+} efflux as a function of time (A), $^{45}Ca^{2+}$-loaded vesicles were resuspended in efflux medium, with or without ether (see Experimental Procedures). Upon the addition of 2 mM ADP, aliquots (150 μl) were then withdrawn at the times indicated and centrifuged to obtain tight SR pellets in 2 min. Net Ca^{2+} efflux was determined from $^{45}Ca^{2+}$ trapped in the pellets; each time point was then corrected for Ca^{2+} leakage by subtracting the amount of passive $^{45}Ca^{2+}$ efflux from controls done in the absence of ADP. For measurements of net ATP synthesis as a function of time (B), "cold" Ca^{2+}-loaded vesicles were resuspended in various efflux media (see Experimental Procedures). The synthesis of γ-^{32}P]ATP was initiated by the addition of 20 mM ($^{32}P_i$ + P_i) and 2 mM ADP; aliquots (0.3 ml) of the efflux reaction mixture were transferred at the times indicated to stop the reaction. Net ATP synthesis was determined from the formation of [^{32}P]glucose-6-phosphate from the newly synthetized [γ-^{32}P]ATP. Each time point was corrected for a specific formation of [^{32}P]glucose-6-phosphate by subtracting the amount of [^{32}P]glucose-6-phosphate measured in parallel controls lacking ADP.

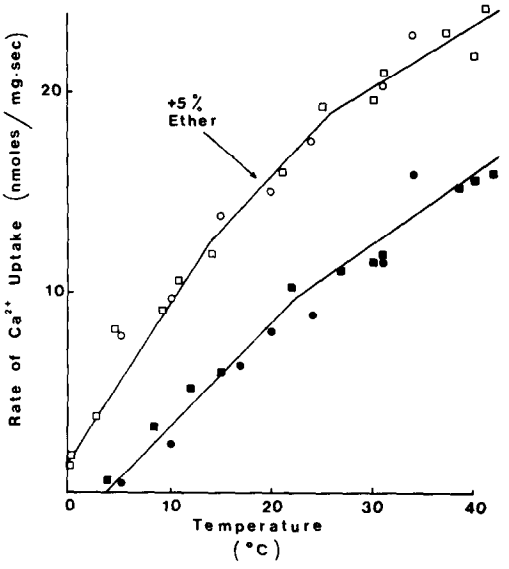

Fig. 6. Rate of Ca^{2+} uptake by SR as a function of temperature. Ca^{2+} uptake by SR was measured through ΔA of arsenazo III (as shown in Fig. 1) at various temperatures in the range of 0–40°. A water bath circulator was used to keep the medium at the desired temperature before the addition of vesicles and to maintain the cuvette holder inside the multiple-wavelength spectrophotometer at the same temperature. The average rate of Ca^{2+} uptake during the first 5 sec of the reaction was plotted as a function of temperature for two SR preparations, in the absence of ether (●, ■) and in the presence of 5% (v/v) ether (○, □). Other experimental conditions were identical to those in Fig. 1.

could be used to detect changes in Ca^{2+} concentrations in the presence of the high concentrations of EGTA necessary for these experiments.

As shown in Fig. 5A, the normal rate of active efflux from Ca^{2+}-loaded SR averaged to 20 nmoles Ca^{2+} per mg protein per min, about twice the rate of ATP synthesis. In the presence of ether, the rate of efflux was enhanced to 45 nmoles Ca^{2+} per mg protein per min during the first 3 min of the reaction and declined as the transmembrane Ca^{2+} gradient collapsed. In diethyl ether, the ratio of outward Ca^{2+} translocation to ATP formed was also approximately 2 to 1, clearly indicating that diethyl ether stimulated the reversal of the Ca^{2+} pump as well as the forward reaction.

Temperature dependence of Ca^{2+} uptake velocity in the presence of diethyl ether. The ether-induced stimulation of Ca^{2+} uptake by SR was measured at various temperatures through the absorption changes of arsenazo III. In the absence of oxalate, the initial rate of Ca^{2+} uptake averaged over the first 5 sec of the reaction was determined from experimental traces similar to those shown in Fig. 1 and was plotted as a function of temperature in Fig. 6. In the absence of ether, Ca^{2+} uptake was completely inhibited below 5°; in the range of 5 to 20°, the velocity of uptake increased by about 0.5 nmole Ca^{2+} per mg protein per sec per °, and from 20 to 40° it increased more slowly by about 0.38 nmole Ca^{2+} per mg protein per sec per °. In the presence of 5% diethyl ether, measurable Ca^{2+} accumulation occured at 0°; the velocity of uptake increased by about 1.0 nmole Ca^{2+} per mg protein per sec per °. In the range of 0 to 20°, and from 20 to 40° it increased more slowly as in the absence of ether by about 0.36 nmole Ca^{2+} per mg protein per sec per °. In both cases at 42° and above (not shown), Ca^{2+} accumulation was rapidly inhibited, in agreement with previous reports of an uncoupling of the SR ATPase due to a thermally-induced protein conformational change [27].

The ether-induced stimulation of Ca^{2+} uptake was considerably more pronounced at low temperatures and decreased steadily in going from 0 to 40°. Ether enhanced the initial rate of Ca^{2+} uptake by a factor of 7.1 at 5°, 3.0 at 10°, 2.0 at 20°, 1.6 at 30° and 1.5 at 40° (Fig. 6). Thus, at low temperatures when the SR membrane is known to exhibit a state of lower entropy and fluidity [28], ether was particularly effective in stimulating Ca^{2+} uptake, while at higher temperatures, states of greater entropy and membrane fluidity already stimulate the Ca^{2+} pump and limit the ability of ether to further enhance the enzyme turnover rate.

Concentration of diethyl ether in the aqueous and lipid phases. During the experiments described above, SR reaction mixtures were kept in open containers exposed to air at room temperatures. Even though ether is highly volatile, SR incubated in ether for periods ranging from 1 to 30 min, in open vials, exhibited similar increases in rates of Ca^{2+} transport, ATPase activity, and total Ca^{2+} accumulation. The extent of ether lost from the medium as a result of evaporation was determined by gas chromatography. As shown in Fig. 7, the concentration of ether (mg ether/ml) in solutions with or without SR was found to decrease by about 10% in 10 min and 30% in 30 min. Thus, for most experiments lasting about

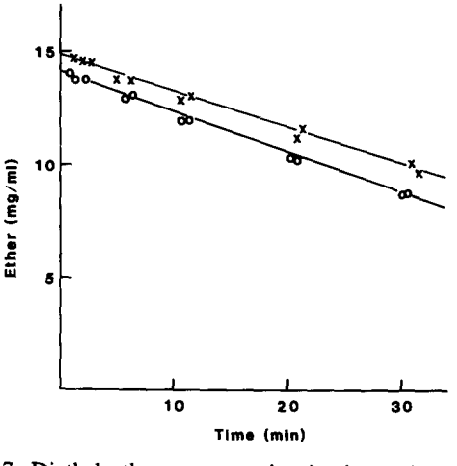

Fig. 7. Diethyl ether concentration in the medium as a function of time. Ether (5%, v/v) was added to reaction mixtures at time t = 0, and its concentration in solution was measured at chosen times by gas chromatography. In the absence (–○–) or presence (–×–) of SR (1 mg protein/ml), the concentration of ether in solution decreased at a rate of about 1%/min due to evaporation. The solutions contained 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–maleate (pH 7.0) at 23°.

Table 1. Diethyl ether concentrations in lipid and aqueous phases of SR

% Ether	Ether (mg/ml H_2O)	Ether concn in supernatant fraction C_{aq} (mole ether / mole H_2O)	Ether (mM)	Ether concn in pellet* (mg/pellet)	Ether concn in water space of pellet† (mg/H_2O of pellet)	Ether‡ (μg)	Ether concn in lipid phase of pellet C_m§ (Ether μmoles / (lipid in pellet))	C_m§ (mole ether / mole lipid)	Partition coefficient $P = C_m/C_{aq}$
	6.72	1.6×10^{-3}	90.6	0.320	0.265	55	0.74	0.025	15.6
1.25	6.23	1.5×10^{-3}	84.0	0.301	0.245	56	0.76	0.025	16.7
	13.0	3.0×10^{-3}	175.4	0.616	0.512	104	1.40	0.045	15.33
2.5	12.1	2.9×10^{-3}	163.3	0.569	0.477	92	1.24	0.041	14.1
	19.6	4.8×10^{-3}	264.4	0.960	0.772	188	2.54	0.085	17.7
5	20.1	4.9×10^{-3}	271.0	0.989	0.792	197	2.66	0.0886	18.1
	33.9	8.2×10^{-3}	457.5	1.662	1.336	326	4.4	0.147	17.9
10	36.2	8.8×10^{-3}	488.4	1.765	1.426	338	4.6	0.153	17.3

* Each pellet contains 7.22 mg SR protein.
† Total ^3H_2O volume of pellets, $V_T = 39.4$ μl pellet.
‡ Total ether contents in pellet – ether in water space of pellet.
§ The lipid to protein ratio in SR was taken as 0.58 mg lipid/mg protein (from Ref. 24), and the average molecular weight of SR lipid was taken to be 140.

10 min, the evaporation of ether was insignificant and had no effect on the experimental results. The concentration of anesthetic in the non-aqueous phase was determined for vesicles equilibrated in 1.25, 2.5, 5, or 10% diethyl ether (Table 1). Values obtained for the partition coefficient of ether between the lipid and water space ranged from 14.1 to 18.1 [(moles ether per mole lipid)/(moles ether per moles H_2O)], in excellent agreement with previously reported values of 16.4 for a similar SR preparation in 5% (v/v) diethyl ether [28]. Under these experimental conditions, the molar ratio of ether to lipid could well account for the swelling of SR vesicles [10, 11]. On the other hand, the persistent stimulation of Ca^{2+} transport 60 min after the addition of ether, that is, despite a significant amount of ether evaporation from the solution, suggests that the interaction of ether with the Ca^{2+}-pump protein and its surrounding lipid is likely to differ from its interaction in the bulk lipid phase.

Reversibility of the effect of ether on the Ca^{2+} *pump.* The stimulation of Ca^{2+} transport and ATPase activity by SR in the presence of ether could be reversed back to normal values by washing the vesicles in an ether-free medium. Vesicles were mixed into reaction media containing ether 0, 2.5, 5 or 10% diethyl ether, equilibrated for 30 min and centrifuged, and the pellets were resuspended gently in ether-free solutions. Precautions were taken to verify that the vesicles in media with or without ether

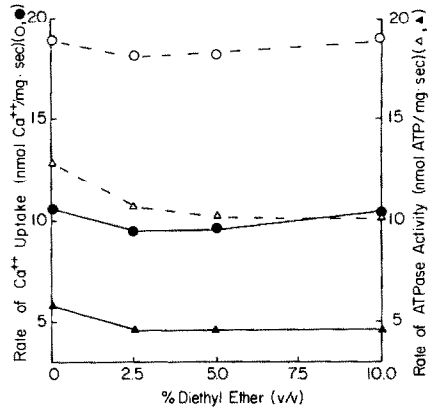

Fig. 8. Rate of Ca^{2+} uptake and ATPase activity of ether-treated SR. Ether treatment: SR were incubated for 30 min in 100 mM KCl, 10 mM $MgCl_2$, 5 mM Tris–maleate (pH 7), in either 0, 2.5, or 10% (v/v) diethyl ether, centrifuged, and then resuspended in ether-free medium. Ca^{2+} uptake and ATP hydrolysis of ether-treated SR were measured simultaneously through ΔA of arsenazo III and the acidification of the medium with a pH electrode as described for Fig. 1. The average rates of Ca^{2+} uptake (●) and ATPase activity (▲) during the first 5 sec of the reaction were measured and plotted as a function of the ether concentration in the initial incubation medium. The rates measured with vesicles treated with 2.5, 5, or 10% ether were similar to each other and to controls (i.e. 0% ether in the incubation medium). The re-addition of 5% ether approximately doubled the rates of Ca^{2+} uptake (○) and ATP hydrolysis (△) of SR treated with either 0, 2.5, 5, or 10% ether. Protein concentrations were measured before and after ether treatment by the method of Lowry *et al.* Temperature was 23°.

could be recovered in the pellets with no significant losses in the supernatant fraction. Assays of protein concentration measured from each sample before and after the centrifugation step showed that 92–96% of the total protein was recovered. As shown in Fig. 8, the initial rates of Ca^{2+} uptake and ATP hydrolysis by SR were not altered significantly by treatment of SR with 2.5, 5 or 10% ether compared to 0% ether. Moreover, the re-addition of 5% ether to ether-treated SR again stimulated both the rates of Ca^{2+} uptake and ATPase activity by about a factor of 2.

Even in the absence of oxalate, the ability of SR to retain their accumulated Ca^{2+} after the depletion of ATP in the medium was not diminished by ether treatment (Fig. 9). Moreover, the second subsequent readdition of 5% diethyl ether doubled the rate of uptake (see Fig. 8) but, once the hydrolysis of ATP was completed, the vesicles were found to be somewhat more leaky and retained less total calcium (Fig. 9) than normal or ether-treated vesicles (Fig. 9). In contrast, previous studies showed that ether treatment rendered SR leaky and incapable of storing Ca^{2+} [7, 8]. In attempts to explain this discrepancy, SR treated with 10% ether were vigorously homogenized and shaken by vortex agitation for 5 sec to determine whether or not ether treatment rendered the vesicles more delicate and prone to lysis by mechanical pertubations. SR treated with 3 or 5% ether and vortexed could still take up Ca^{2+} at rates comparable to normal vesicles and still retained significant concentrations of their sequestered Ca^{2+} after the depletion of ATP (Fig. 9). On the other hand, the same mechanical pertubations applied to SR kept in a 10% ether medium were considerably

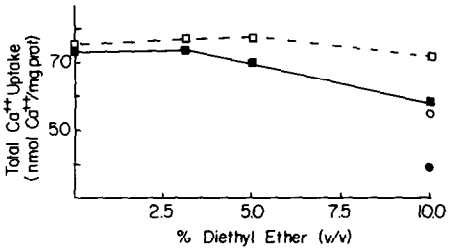

Fig. 9. Total Ca^{2+} uptake by ether-treated SR. The maximum amounts of Ca^{2+} sequestered (--□--) by SR treated with 0, 3, 5, or 10% ether were quantitatively measured through ΔA of arsenazo III, in the absence of oxalate or other Ca^{2+} precipitating agents. The medium consisted of 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM Tris–maleate (pH 7.0), 0.1 mM arsenazo III, 0.5 mg SR protein/ml, and 1 mM ATP. The total Ca^{2+} sequestered by ether-treated SR was similar to controls (--□--, 0% ether-treated SR). The addition of 5% ether to vesicles already treated with ether reduced their ability to store calcium (-■-). Mechanical disruption of SR by vortex agitation for 5 sec slightly reduced the Ca^{2+} uptake of 10% ether-treated vesicles (○), but vortex agitation of vesicles incubated in 10% ether was considerably more effective in breaking up the vesicles (●). Temperature was 23°.

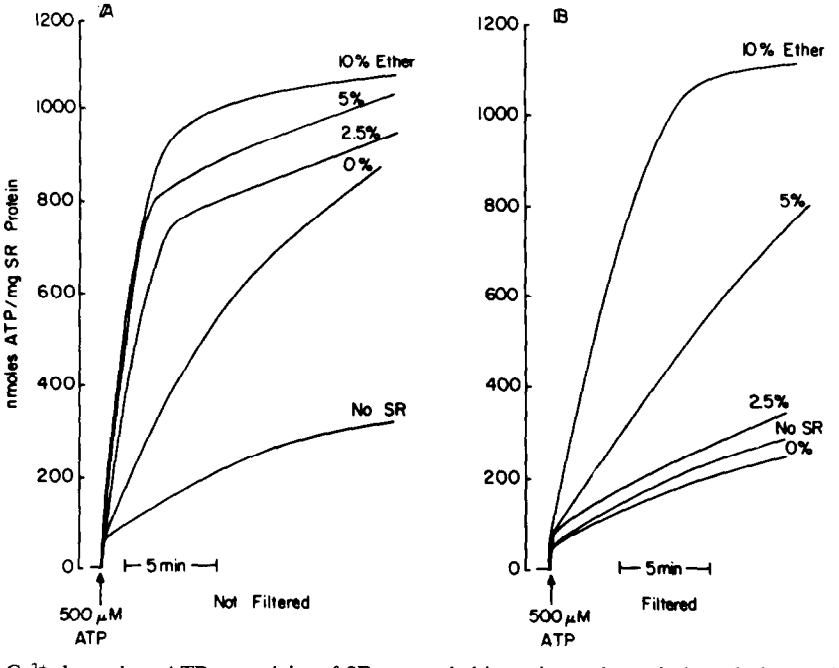

Fig. 10. Ca^{2+}-dependent ATPase activity of SR suspended in various ether solutions, before and after Millipore filtration. Ca^{2+}-dependent ATPase activity of SR was measured through the acidification of the reaction mixture before (A) or after (B) Millipore filtration. The reaction mixture (10 ml) consisted of 0.15 mg SR protein/ml, 100 mM KCl, 10 mM $MgCl_2$, 2.5 mM Ca^{2+}–EGTA, 5 mM Tris–maleate (pH 7.0), and either 0, 2.5, 5, or 10% ether. In panel A, ATP hydrolysis was measured upon the addition of 0.5 mM ATP in 2-ml aliquots of the reaction mixture. In panel B, a 2-ml aliquot was filtered through a 0.45 μm Millipore filter (2.5 cm in diameter), and the ATPase activity of the filtrate was then measured upon the addition of 0.5 mM ATP. Temperature was 23°.

more effective in disrupting the vesicles and markedly reduced their ability to store calcium (Fig. 9).

Separation of SR vesicles by Millipore filtration. Measurements of $^{45}Ca^{2+}$ uptake by SR using standard Millipore filtration techniques depend on the rapid filtration of the SR suspensions and on the thorough trapping of the vesicles by the filters. The ability of Millipore filters (0.45 μm pores) to trap SR suspended in ether solutions was tested by monitoring the ATPase activity of suspensions (1 ml) before filtrations and comparing it to the residual activity found in filtrates. In typical experiments with no ether, 0.1 mg SR protein was trapped completely by 0.45 μm Millipore filters with surface areas of 4.9 cm^2 as demonstrated by the low ATPase activity in the filtrate (Fig. 10A and B, compare traces for 0% ether). Increasing ether concentrations enhanced the activity of the Ca^{2+} pump measured from unfiltered reaction media (Fig. 10A); however, the ATPase activity of the filtrates was no longer negligible. As shown in Fig. 10B, the ATPase activity of the filtrates increased with the ether concentration in the medium, and at 10% diethyl ether the rate of ATP hydrolysis in the filtrate was nearly equal to that in the unfiltered suspension. Consequently, with ether present in the solution, Ca^{2+} transport by SR could not be measured by standard Millipore filtration techniques since the filters did not trap the vesicles.

Dimensions of SR suspended in ether solutions. Previous qualitative observations on the structure of SR by electron microscopy suggested that ether treatment alters the morphological appearance of the vesicles. Non-treated SR preparations consisted of tubular elements often delimited by a double-layer membrane, while ether-treated preparations were almost totally replaced by swollen, ovoid vesicles possessing continuous, but thinner, walls which often seemed to be fused [7, 29]. The morphological appearance of membranes from treated SR preparations was taken to be in harmony with the premise that treated vesicles become leaky and incapable of accumulating calcium. However, the incubation of SR with ferritin revealed that very few ferritin granules penetrated into the lumen of the vesicles indicating that, if ether produced holes in SR membrane, the holes would have to be smaller than 110 Å [29].

In more recent experiments, intravesicular volumes measured through the total (^3H_2O) tritiated water volume minus the excluded [^{14}C]polydextran volume of SR pellets indicated that the presence of 5% ether increases the internal volume of the vesicles by about 40% and does not render the vesicles leaky [10]. The morphological alterations induced by ether were re-examined by electron microscopy. For SR incubated in 5% ether, no alterations in the shape or in the appearance of the membrane were detected; however, there was a noticeable increase in the size of the vesicles. The changes in particle size induced by ether were quantitatively determined with an image analyzer (OMNICON, Bausch & Lomb). As shown in the histograms (Fig. 11), the mean diameter of vesicles exposed to 5% ether (A) was approximately 20% greater than normal (B) which is equivalent to a 70% increase in intravesicular volume. The larger vesicles appeared to be neither lysed nor fused to neighboring vesicles.

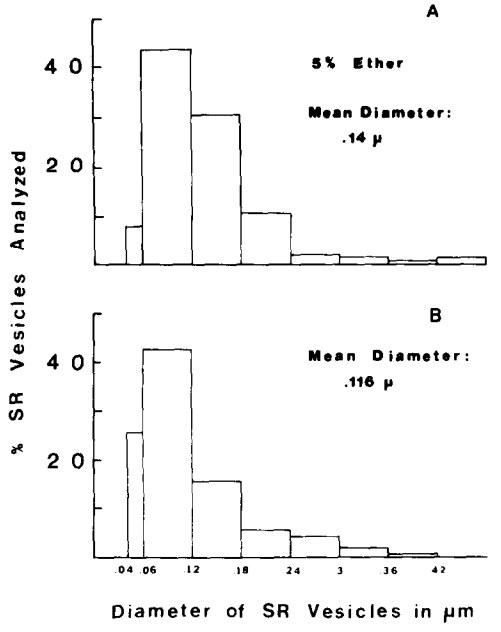

Fig. 11. Mean diameter of SR in the presence or absence of 5% diethyl ether. SR were incubated for 20 min in 100 mM KCL, 10 mM $MgCl_2$, 20 mM Tris–maleate (pH 7.0), 0.2 mM $CaCl_2$, at 23° with or without 5% diethyl ether, and then centrifuged (98,000 $g \times 2$ min) with an airfuge to form tight SR pellets. After staining the pellets (see Experimental Procedures), five electromicrographs from central cross-sections of each pellet were taken and scanned with an OMNICON image analyzer to determine the distribution diameter of SR incubated with (A) or without (B) diethyl ether. Mean diameters were calculated from approximately 1000 vesicles in the image field; the algorithm excluded particles less than 0.04 μm in diameter caused by specks on the film.

DISCUSSION

The results of this study unequivocally indicate that diethyl ether enhances active Ca^{2+} uptake, Ca^{2+}-dependent ATPase activity, and the total Ca^{2+} capacity of actively loaded SR but inhibits markedly the Ca^{2+}-independent or "basal" ATPase activity of SR preparations. These experimental observations were highly reproducible and were clearly demonstrated for a wide range of SR, diethyl ether, Ca^{2+}, and ATP concentrations in the reaction medium. Ether was also found to enlarge the internal volume of SR, stimulate Ca^{2+} efflux coupled to ATP synthesis through the reversal of the Ca^{2+} pump, and markedly enhance Ca^{2+} uptake by SR at low temperatures (0–10°), at which Ca^{2+} transport is normally negligible or absent. Moreover, the stimulation of active Ca^{2+} transport by SR, induced by the presence of ether, could be largely reversed by washing the vesicles in ether-free solutions. Consequently, "ether-treated" SR (a term first introduced by Inesi *et al.* [7]), that is, SR incubated in diethyl ether, centrifuged, and then gently resuspended in ether-free media, were found to have normal Ca^{2+}-trans-

port properties like those of SR that had never been exposed to diethyl ether. Put together, these results strongly suggest that the primary mode of action of diethyl ether is to enhance the fluidity of the SR membrane, which increases the forward and reverse turnover rates of the Ca^{2+} pump and enlarges the volume of vesicles so that, with respect to controls, greater amounts of Ca^{2+}, yet similar concentrations, are stored in the lumen of vesicles with larger intravesicular space.

As we reported previously [10, 11], these results are in sharp contrast with earlier observations [7, 8, 29] that the presence of diethyl ether, or the "ether treatment" of SR drastically depresses active Ca^{2+} transport. Except for our initial communication, virtually all the experimental evidence obtained with isolated SR from various laboratories shows that the presence or treatment of SR with 5–10% diethyl ether increases Ca^{2+}-dependent ATPase activity, inhibits or abolishes ATP-dependent Ca^{2+} transport, and enhances passive Ca^{2+} efflux from Ca^{2+}-loaded SR, but it does not alter "basal" ATPase activity. These observations have been interpreted as indicating an ether-induced increase in the Ca^{2+} permeability of the SR membrane which short-circuits the sequestered calcium and makes the vesicles incapable of accumulating or maintaining high Ca^{2+} concentrations in the intravesicular space. Consequently, the Ca^{2+}-dependent ATPase activity of "ether-treated" SR was thought to be uncoupled from the calcium uptake process, and the enhanced rates of ATP hydrolysis were attributed to the levels of calcium remaining in the reaction medium since the vesicles were rendered "leaky" and could no longer actively sequester Ca^{2+}.

The present experiments show that these major discrepancies can be attributed to either the procedures used to prepare "ether-treated" SR or the methods used to measure Ca^{2+} transport. In some previously reported experiments [7], ether treatment of SR consisted of an incubation in ether solutions (2.5 to 25%, v/v), centrifugation, and resuspension in ether-free solutions, which resulted in leaky or lysed vesicles incapable of Ca^{2+} accumulation. The combination of these three steps plus the long centrifugation time necessary to pellet the vesicles could have led to irreversible damage of the SR. However, as shown in Fig. 8, "ether treatment" by a similar procedure had little or no effect on the rates of calcium uptake and ATP hydrolysis. On the other hand, SR in ether solutions and "ether-treated" vesicles became more fragile and more susceptible to irreversible lysis by mechanical disruption with a vortex agitator (Fig. 9). "Ether treatment" by iteself did not lyse SR but, in earlier studies [7], SR were typically stored, ether-treated in hypotonic 5 mM Tris–maleate solutions, and then diluted in 250 mOsM KCl buffers for measurements of Ca^{2+} uptake. While normal SR can withstand these osmotic changes, the more fragile "ether-treated" vesicles could well be broken by a combination of mechanical and osmotic forces. In other published experiments [8], ATP-dependent Ca^{2+} uptake and passive Ca^{2+} efflux by SR in the presence and absence of diethyl ether were measured radiochemically through the distribution of $^{45}Ca^{2+}$ in the SR and in the reaction mixture after filtration. However, the routine application of this method to measure Ca^{2+} transport produces serious artifacts because the presence of diethyl ether in the medium alters the characteristics of the filters and dramatically reduces their ability to trap the vesicles. Control experiments aimed at demonstrating this possibility were carried out through measurements of Ca^{2+}-dependent ATPase activity in SR reaction mixtures containing diethyl ether (0–10%, v/v) before (Fig. 10A) and after (Fig. 10B) Millipore filtration. In the absence of ether, ATPase activity in the filtrate was quickly diminished since the filters trapped the SR effectively, whereas in the presence of increasing concentrations of ether the ATPase activity in the filtrate was nearly equal to its activity measured in non-filtered SR suspensions. This indicates that, in the presence of ether, SR are not retained by the filters. Thus, active Ca^{2+} uptake, measured through the $^{45}Ca^{2+}$ counts associated with SR presumably trapped on the filters, is greatly underestimated since, in ether, the vesicles appear in the filtrate, which leads to the implication that diethyl ether significantly reduces Ca^{2+} uptake by SR.

In general, volatile anesthetics are known to perturb the structure of lipid bilayers by altering lipid–lipid interactions and can also increase the rotational and lateral diffusion rates of various molecules in the membrane [28]. The various changes in the properties of lipid bilayers induced by such agents have been attributed to increases in "membrane fluidity", a single term used to describe altered lipid–lipid interactions. In the presence of diethyl ether (8%, v/v), the melting point of phospholipids and of SR membranes was shifted to lower temperatures. The shift in phase transition induced by ether accounts for increases in passive diffusion of molecules embedded in the membranes [28] and for the expansion of the surface area of lipid bilayers by amouts greater than that predicted from the concentration of ether incorporated in the membrane [30, 31]. The incubation of liposomes in ether results in an expansion of membrane and internal trapping volume of the vesicles [32] but only slight increases in K^+ and Na^+ permeabilities [31]. Large liposomes with a volume trapping efficiency ten times that of standard sonicated or hand-shaken preparations have been obtained by an ether injection technique [33]. These vesicles with particularly large internal volumes were mostly unilamellar, and the residual ether in their membrane did not affect their permeability to cations or their osmotic permeability to small uncharged molecules [33]. In recent experiments on crayfish giant axons, ether produced a dose-dependent, reversible increase in the rates of sodium current inactivation and potassium current activation at all membrane potentials [34]. Speeding up the kinetics of sodium and potassium gating channels by ether was consistent with the hypothesis that the rates of gating processes are dependent on the "fluidity" of the lipids surrounding the channels [34].

The experiments presented here strongly suggest that the ether-induced stimulation of the SR Ca^{2+} pump is caused by altered lipid to protein interactions which, in turn, permits the faster translocation and

release of Ca^{2+} in the lumen of the SR, which appears to be the rate-limiting step in the recycling of the enzyme. Morphological studies by electron microscopy [7, 29], radiochemical measurements of intravesicular volume [10], and the present morphometric analysis of average vesicular diameter (Fig. 11) all indicate that diethyl ether also enlarges the intravesicular volume of SR. As a result, larger amounts of Ca^{2+} must be sequestered in the larger SR vesicles exposed to ether to obtain intravesicular Ca^{2+} concentrations similar to control SR. The large partition coefficient of ether in the lipid phase can account for the increased intravesicular space of SR in ether and for the extra total Ca^{2+} accumulation; however, it does not explain increases in the velocity of Ca^{2+}-dependent ATPase activity.

At low temperatures, the rate of Ca^{2+} uptake by SR was reduced markedly or blocked completely primarily because the viscosity of the membrane inhibited the translocation of Ca^{2+}. The presence of ether, which is known to increase the fluidity of the SR membrane [28], overcame this inhibition and increased significantly the rates of transport at temperature ranging from 0 to 40°. Moreover, ether was particularly effective at low temperatures (i.e. low membrane fluidity) compared to high temperatures (i.e. already high membrane fluidity). Consistent with the view that ether enhances the turnover rate of the Ca^{2+} pump by altering the lipid to protein ratio, it also increased the velocity of the reverse process of Ca^{2+} efflux coupled to ATP synthesis (Fig. 5), Ca^{2+} transport by reconstituted SR, and could be effectively washed out by resuspending the vesicles in ether-free solutions (Figs. 8 and 9). Alternative mechanisms were also considered to explain the ether-induced increase in the rate of transport: (a) an increase in Ca^{2+} to ATP coupling ratio, and (b) the "exposure" of a greater population of ATPases or Ca^{2+} pumps within the membane. Ca^{2+} transport measurements using arsenazo III (Fig. 1) and rapid kinetic measurements of the initial phase of the reaction by stop-flow techniques [10] indicated that ether produced a 2-fold increase in the maximal levels of both Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity. Furthermore, at various ether concentrations, increases in Ca^{2+}-dependent ATPase activity paralleled increases in Ca^{2+} uptake such that an ether-induced change in the degree of coupling and Ca^{2+} to ATP ratio is unlikely to occur. An increase in the population of ATPases in the membrane that is exposed and available for the translocation of Ca^{2+} is not likely to occur since steady-state levels of phosphoenzyme formation (E-P) were not altered by the presence of ether (not shown).

The inhibition of Ca^{2+}-independent or basal ATPase activity by ether suggests a more complex mode of action, perhaps through a direct interaction between the anesthetic and the ATPase. On the other hand, there is a growing consensus in the literature that basal ATPase activity arises from a separate enzyme, a prevalent contaminant of SR preparations distinguishable from the Ca^{2+} pump. For instance, a fraction of heavy SR, free of basal ATPase activity, yet efficient at Ca^{2+} pumping, has been isolated by a lengthy novel procedure [26]. Further, experiments have shown that the Ca^{2+}-dependent SR ATPase can catalyze the hydrolysis of (ATP-β-S), an analogue of ATP adenosine 5 - *O*-(2-thiotriphosphate) at rates and concentration identical to those of ATP in the presence of Ca^{2+} but that ATP-β-S was not a substrate for the Ca^{2+}-independent ATPase [35].

The data presented here are in line with physiological studies on intact muscle and account for the observed relaxation of muscles bathed in ether solution.

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