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Thapsigargin and Dimethyl Sulfoxide Activate Medium $P_i \leftrightarrow HOH$ Oxygen Exchange Catalyzed by Sarcoplasmic Reticulum Ca^{2+}-ATPase*

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Thapsigargin is a potent inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase. It binds the Ca^{2+}-free E2 conformation in the picomolar range, supposedly resulting in a largely catalytically inactive species. We now find that thapsigargin has little effect on medium $P_i \leftrightarrow HOH$ oxygen exchange and that this activity is greatly stimulated (up to 30-fold) in the presence of 30% (v/v) Me_2SO. Assuming a simple two-step mechanism, we have evaluated the effect of thapsigargin and Me_2SO on the four rate constants governing the reaction of P_i with Ca^{2+}-ATPase. The principal effect of thapsigargin alone is to stimulate *E*P hydrolysis (k_{-2}), whereas that of Me_2SO is to greatly retard P_i dissociation (k_{-1}), accounting for its well known effect on increasing the apparent affinity for P_i. These effects persist when the agents are used in combination and substantially account for the activated oxygen exchange ($v_{exchange} = k_{-2}[EP]$). Kinetic simulations show that the overall rate constant for the formation of *E*P is very fast (~300 s^{-1}) when the exchange is maximal. Thapsigargin greatly stabilizes Ca^{2+}-ATPase against denaturation in detergent in the absence of Ca^{2+}, as revealed by glutaraldehyde cross-linking, suggesting that the membrane helices lock together. It seems that the reactions at the phosphorylation site, associated with the activated exchange reaction, are occurring without much movement of the transport site helices, and we suggest that they may be associated solely with an occluded H^+ state.

Sarcoplasmic reticulum (SR)[1] Ca^{2+}-ATPase pumps Ca^{2+} from the sarcoplasm to the reticular lumen to allow relaxation in skeletal muscle. Transport of Ca^{2+} is driven by ATP hydrolysis, and the catalytic cycle includes phosphorylation and dephosphorylation of an aspartic acid residue. The pump can operate in the reverse direction, coupling P_i phosphorylation and phosphoryl transfer to ADP with Ca^{2+} efflux down the concentration gradient. Chemical reactions at the active site depend on whether the transport sites are occupied with Ca^{2+} (E1 forms, reactive to ATP or ADP) or H^+ (E2 forms, reactive to P_i and water) ions and their orientation with respect to the membrane.

The reaction with P_i, in its simplest representation, can be described by two steps, shown in Scheme 1. Mg^{2+} and P_i either bind independently (1, 2) or as MgP_i (3).

$$E2 + Mg^{2+} + P_i \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} E2 \cdot Mg{\cdot}P_i \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} E2 - P{\cdot}Mg + H_2O$$

SCHEME 1

It is well known that the phosphorylation reaction is augmented by co-solvents like Me_2SO and glycerol, by greatly increasing the apparent affinity for P_i (3–7). The rate constants in Scheme 1 changed by Me_2SO have not been fully elucidated. Although dephosphorylation has been found to be inhibited (3, 4), the k_{obs} for phosphorylation is activated in the 0–20% (v/v) Me_2SO concentration range (3) and strongly inhibited at 40% (4). de Meis *et al.* (4) consider that the main effect may be a true affinity change caused by decreased solubility of P_i in the medium and favored partitioning of P_i into the catalytic site. In contrast, Mintz *et al.* (8) consider that their competition studies of $[^{45}Ca]Ca^{2+}$ and P_i binding show that the apparent affinity change is due to an effect on the covalent phosphorylation reaction itself.

Thapsigargin, a sesquiterpene lactone isolated from the plant *Thapsia garganica*, is a specific inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPase isoforms (9–12). It binds the E2 conformation of SR Ca^+-ATPase extremely tightly in a 1:1 molar ratio (13–16). Site- and segment-directed and natural mutagenesis has shown that thapsigargin binding is sensitive to amino acid changes in transmembrane helix 3, and Phe-256 and Gly-257 in this helix are especially critical (17–21). Photolabeling with an azido analogue of thapsigargin located the site in the protein section between stalks S3 and S4 (22). More recently, three-dimensional reconstruction of images obtained by cryo-electron microscopy of tubular crystals suggests that thapsigargin binds to protein luminal loops between transmembrane segments M3/M4 and M7/M8 (23). Thapsigargin binding inhibits Ca^{2+}-dependent ATP phosphorylation as well as P_i phosphorylation and reportedly results in a largely catalytically inactive complex (13–15).

During the course of the equilibrium phosphorylation reaction depicted in Scheme 1, the four oxygens of medium P_i are necessarily exchanged with those in water. If P_i enriched in ^{18}O is used, then the ^{18}O atoms are gradually lost to water and ^{16}O-substituted. In addition to the obligatory replacement of at least one oxygen atom per phosphorylation and dephosphoryl-

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; *E*P, phosphoenzyme; FITC, fluorescein 5′-isothiocyanate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TMAH, tetramethylammonium hydroxide; MES, 2-(*N*-morpholino)ethanesulfonic acid; E1, form of Ca^{2+}-ATPase in the presence of Ca^{2+}; E2, form of Ca^{2+}-ATPase in the absence of Ca^{2+}.

ation event, there may be more because of dynamic reversal of bound P_i to phosphoenzyme formation before the P_i is released to the medium. SR Ca^{2+}-ATPase catalyzes a rapid medium[2] $P_i \leftrightarrow$ HOH oxygen exchange (24, 25). Analysis of the development of the five P_i species with differing amounts of $[^{18}O]P_i$ allows, in combination with phosphoenzyme measurements, estimation of the average number of reversals of bound P_i and elucidation of all four rate constants of the reaction (3, 26, 27).

In this study, we find that thapsigargin binding has little effect on $P_i \leftrightarrow$ HOH oxygen exchange and that Me_2SO greatly accelerates this activity. The basis for the effect is sought through evaluating the four rate constants of the P_i reaction. The results describe the effects of thapsigargin and Me_2SO, alone and in combination, on these rate constants at pH 6.0 and 7.0. Further, it is shown that thapsigargin greatly stabilizes the Ca^{2+}-free Ca^{2+}-ATPase against denaturation in detergent, suggesting that the membrane helices are locked in an immobilized state. The unexpected activation of catalytic events in the stable thapsigargin-Ca^{2+}-ATPase complex has important implications for the coupling of these catalytic reactions with H^+ transport events at this stage of the cycle.

EXPERIMENTAL PROCEDURES

Materials—$[^{18}O]H_2O$ (99.8 atom %; Amersham Pharmacia Biotech) was used to synthesize highly enriched $[^{18}O]KH_2PO_4$ from PCl_5 (Aldrich) as described by Hackney *et al.* (28). Thapsigargin, ethylamine, and FITC were from Sigma. Me_2SO and diethyl ether were from Merck. Quicksafe A scintillant was obtained from Zinsser Analytic, and $[^{32}P]P_i$ was from Amersham Pharmacia Biotech. The $[^{32}P]P_i$ was purified on a small column of AG 1-X4 (100–200) mesh (Bio-Rad) by eluting with 30 mM HCl. Sodium nitrite, 2-ethoxyethanol, and *p*-toluene-sulfonyl chloride were from Aldrich, and dichloroethane was from Kanto Chemical Co.

SR Vesicle Preparation—SR vesicles were prepared from rabbit muscles according to the method of Champeil *et al.* (3). Amylase (Roche Molecular Biochemicals) was added to the preparation to reduce the amount of phosphorylase contamination (29). Protein concentrations were determined at 280 nm in 50 mM sodium phosphate, pH 7.0, 1% (w/v) sodium dodecyl sulfate. Absorbance was converted into milligrams of protein using a conversion factor of 1, which is based on an experimentally determined relationship between absorbance and SR protein concentration dependence according to the Lowry procedure with bovine serum albumin as a standard. SR preparations used in this study were selected for their high phosphoenzyme levels.

Ca^{2+}-ATPase Content—The amount of active Ca^{2+}-ATPase in the SR preparations, (E_{total}), is often taken as the level of E2P formed from P_i in Me_2SO, which assumes that K_2, the equilibrium constant for the covalent step in the P_i reaction (k_{-2}/k_2), is very small and the equilibrium virtually entirely over to E2P (30). We attempted to measure E_{total} independently of E2P by measuring the amount of Ca^{2+}-ATPase reactive to FITC. This reaction is highly specific for Lys-515 in the nucleotide binding domain (31, 32), and in our experience only a single derivatized peptide is produced following extensive trypsin digestion of the modified protein (33). The FITC concentration dependence of the reaction exhibits a fairly sharp change in slope at approximately the point where all the sites can be expected to be titrated (32), and this concentration may correspond to E_{total}, assuming that all FITC reactive protein is active. Possible error from latter assumption is diminished if preparations are selected that have a very high level of phosphoenzyme, *i.e.* where the proportion of inactive ATPase protein is very small.

Quantification of FITC derivatization was performed as described by Champeil *et al.* (32). SR vesicles (2 mg of protein/ml) were incubated with increasing FITC concentration at 25 °C for 20 min in labeling buffer comprising 100 mM ammonium bicarbonate, pH 7.8, 1 mM $MgCl_2$, 0.01 mM $CaCl_2$, 0.3 M sucrose. FITC solution (2 mM) was made with dimethyl formamide and used immediately. The reaction was stopped by a 10-fold dilution with ice-cold labeling buffer. The samples were centrifuged at 0 °C for 30 min at 150,000 × *g* to pellet SR protein, and the absorbance of the supernatant was measured at 490 nm. The absorbances of standard FITC solutions, without added SR protein, were used for calculating the amounts of unreacted FITC. The stock FITC was found to be greater than 97% pure, and under the reaction conditions chosen, more than 97% of the FITC was reactive to SR vesicles as judged by the disappearance of the FITC peak measured by high performance liquid chromatography (elution monitored at 490 nm).

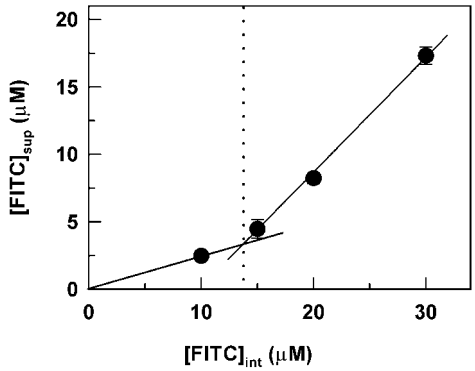


FIG. 1. **FITC titration of Ca^{2+}-ATPase.** SR vesicles (2 mg of protein/ml) were incubated with increasing concentrations of FITC at 25 °C for 20 min in 100 mM ammonium bicarbonate, 1 mM $MgCl_2$, 0.01 mM $CaCl_2$, and 0.3 M sucrose and then centrifuged, and the concentration of FITC was determined in the supernatant. See text for further details. The data points are the averages ± S.D. of five experiments. The sizes of the standard deviations are mostly smaller than the sizes of the *symbols*.

The results are shown in Fig. 1. We performed the assay at four concentrations of FITC, one below the level of EP_{max} (6.3 nmol/mg of protein for this preparation) and three above. More concentrations were not used because the assay required a large amount of protein, and it was important for calculation of K_1 and K_2 that E2P measurements and oxygen exchange were, as far as possible, performed on the same preparation. Preliminary experiments on other preparations showed that there was a linear relationship between concentration of FITC remaining in the supernatant after centrifugation and initial concentration of FITC at low reagent concentrations, below EP_{max}. This permits a straight line to be drawn through the origin and the data point at 10 μM initial FITC. It intersects the line corresponding to excess FITC at 13.8 μM initial FITC, which means that E_{total} equals 6.9 nmol/mg of protein. This is approximately equal to the amount of Ca^{2+}-ATPase (molecular mass, 110 kDa) that can be expected to be present in the preparation if the pump represents 77% of the total protein. The latter value is approximately that found from analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis by the Laemmli method (34) and protein staining with Coomassie Blue (not shown).

SR Ca^{2+}-ATPase Phosphorylation—Stock P_i solutions were made from H_3PO_4, and the concentration was determined by analysis in our chemical pathology diagnostic laboratory at Groote Schuur Hospital, which uses a kit from Roche Molecular Biochemicals based on the standard phosphomolybdate reaction and is measured on an automated Roche/Hitachi analyzer.

Phosphoenzyme formation at 25 °C with $[^{32}P]P_i$ under conditions described in the legends to Figs. 4 and 5 were quenched after 1 min with 5-fold volumes ice-cold 4% (w/v) trichloroacetic acid, 4 mM H_3PO_4. The quenched solutions were allowed to stand for 15 min on ice and then filtered on glass fiber filters (Whatman, GF/F) under a mild vacuum. The filters were extensively washed with ice-cold 4% (w/v) trichlororacetic acid, 2 mM H_3PO_4. Blanks containing 2 mM EDTA instead of 10 mM $MgCl_2$ and 1 mM EGTA were subtracted as background. These blanks were found to be comparable with those obtained by adding the protein to the quench prior to $[^{32}P]P_i$ addition, and vice versa, in the presence of $MgCl_2$ and EGTA. The filters were analyzed for radioactivity after suspending in 5 ml of Quicksafe A scintillant.

Medium $P_i \leftrightarrow$ HOH Exchange—SR vesicles (0.4 mg of protein/ml) were incubated with $[^{18}O]KH_2PO_4$ under conditions described in the legends to Figs. 2 and 3. The reaction was stopped at timed intervals by adding a quarter volume of chloroform and vortexing vigorously. Samples were loaded onto an anion exchange column (2 × 0.5 cm AG1-X4 resin) and washed with water, and the phosphate was eluted with 30 mM HCl (28). The phosphoric acid was lyophilized and ethylated with diazoethane in ether. Diazoethane was generated from *N*-ethyl-*N*-nitroso-*p*-toluene sulfonamide and KOH (35). The sulfonamide was synthesized as described in this reference. Following the ethylation reac-

[2] The word "medium" is used here to contrast this exchange with intermediate $P_i \leftrightarrow$ HOH oxygen exchange, where the P_i produced comes from a different chemical form, namely ATP.

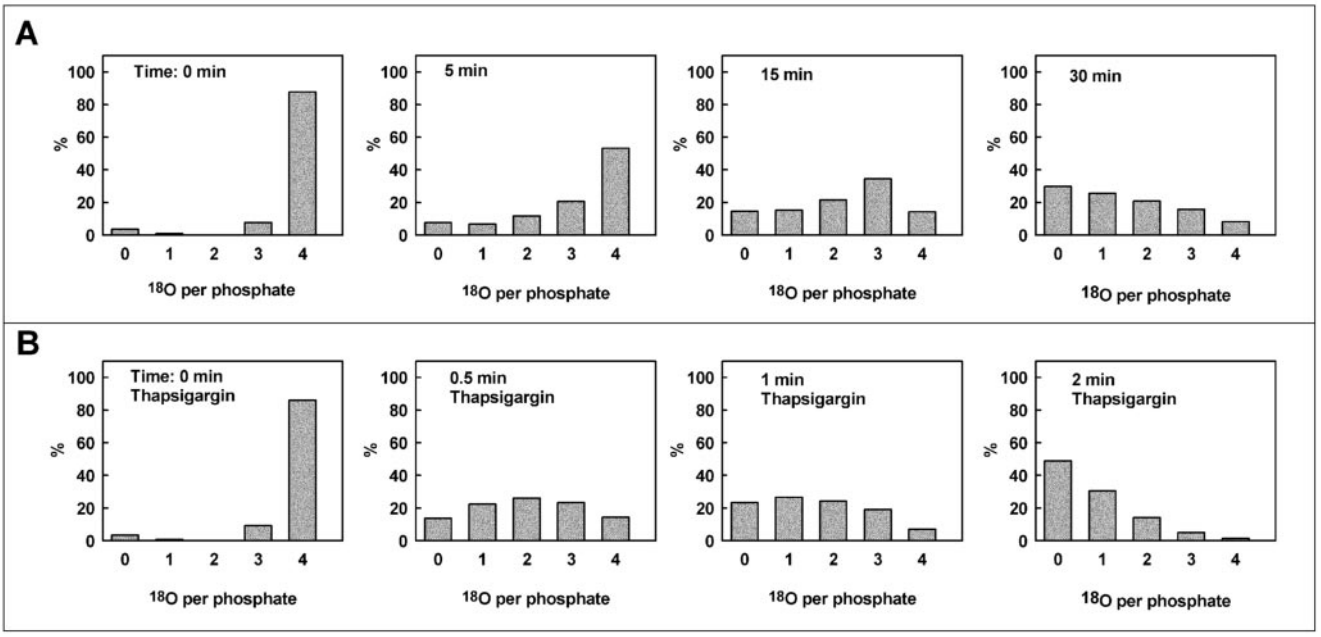


FIG. 2. **Stimulation of medium $P_i \leftrightarrow$ HOH oxygen exchange by thapsigargin.** Oxygen exchange reactions were carried out at 25 °C with SR vesicles (0.4 mg of protein/ml) in 50 mM MOPS/TMAH, pH 7.0, 10 mM $MgCl_2$, 30% (v/v) Me_2SO, 1 mM EGTA, 2 mM [^{18}O]KH_2PO_4, and without thapsigargin (*A*) or with thapsigargin (2 mol/mol of Ca^{2+}-ATPase) (*B*).

tion, the samples were evaporated to dryness, and the triethyl phosphate derivative was dissolved in 50–200 μl of 1,2-dichloromethane for gas chromatography-mass spectrometry. The samples were analyzed with a Hewlett-Packard 5890 GC-5971A mass spectrometer equipped with an HP-1MS capillary column. Masses (99, 101, 103, 105, and 107 kDa) were quantified, and the distribution was expressed as a percentage of all five species. No significant nonphosphate compounds overlapped with these species. Natural abundance [^{18}O]P_i was taken as 0.8% of [^{16}O]P_i, and this amount was subtracted from the *m/z* 101 abundance (28).

Glutaraldehyde Cross-linking—The stability of Ca^{2+}-ATPase was assessed by glutaraldehyde cross-linking (36, 37). The vesicles were preincubated for 15 min at 25 °C in the medium indicated in Fig. 6 before reacting with glutaraldehyde for 5 min. The reaction was stopped by transferring the samples into a 1/10 volume of 150 mM β-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, and a trace of bromphenol blue. The protein solutions were electrophoresed in a 7% acrylamide gel as described by Laemmli (34). The protein bands were stained with Coomassie Blue.

Simulations—Computer simulations of the kinetics of phosphorylation were performed using an iteration program developed in Microsoft Excel.

RESULTS AND DISCUSSION

Effect of Thapsigargin and Me_2SO on Medium Oxygen $P_i \leftrightarrow$ HOH Exchange—The time-dependent change in the profile of medium [^{18}O]P_i species during incubation with control SR vesicles at pH 7.0 in the presence of Me_2SO is shown in Fig. 2*A*. At zero time the predominant species is P_i enriched with 4 atoms of ^{18}O. After 5 min the percentage of this species is less, and species with 3, 2, 1 and zero ^{18}O atoms become more significant, and by 30 min the latter species (with all oxygen atoms ^{16}O) is the most plentiful. The dramatic effect of adding thapsigargin under these conditions is shown in Fig. 2*B*, where it can be seen that after 1 min the distribution of species is rather similar to that in the absence of thapsigargin at 30 min. Thus, thapsigargin stimulates the overall exchange ~30-fold in 30% (v/v) Me_2SO at pH 7.0.

The effect of thapsigargin on the exchange under different conditions can best be compared by plotting the time course for the first order disappearance of $P^{18}O_4$, the species containing four ^{18}O atoms, and the results are shown in Fig. 3. In the absence of Me_2SO, thapsigargin slightly increases the rate

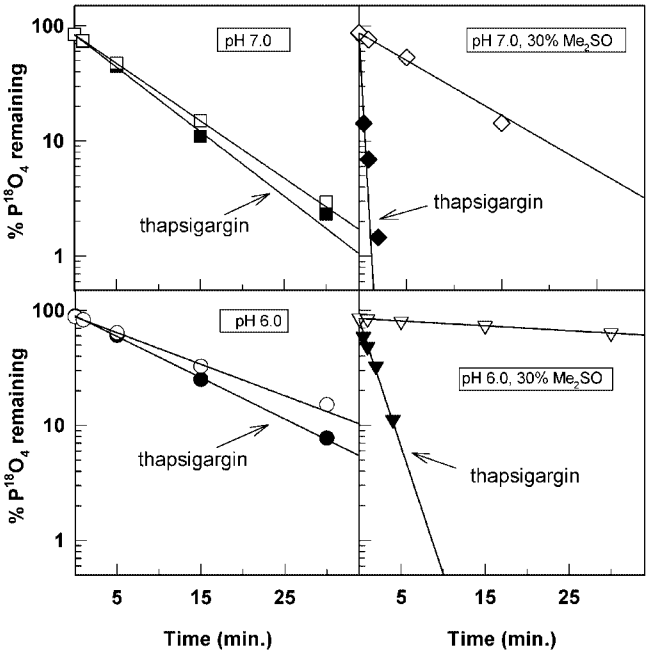


FIG. 3. **Effects of pH, Me_2SO, and thapsigargin on the rate of loss of $P^{18}O_4$ species.** SR vesicles (0.4 mg of protein/ml) were incubated at 25 °C in 50 mM MOPS/TMAH, pH 7.0, 10 mM $MgCl_2$, 1 mM EGTA, and 2 mM [^{18}O]KH_2PO_4 (*upper left panel*); 50 mM MOPS/TMAH, pH 7.0, 10 mM $MgCl_2$, 30% (v/v) Me_2SO, 1 mM EGTA, and 2 mM [^{18}O]KH_2PO_4 (*upper right panel*); 50 mM MES/Tris, pH 6.0, 20 mM $MgCl_2$, 1 mM EGTA, and 2 mM [^{18}O]KH_2PO_4 (*lower left panel*); and 50 mM MES/Tris, pH 6.0, 20 mM $MgCl_2$, 30% (v/v) Me_2SO, 1 mM EGTA, and 2 mM [^{18}O]KH_2PO_4 (*lower right panel*) without thapsigargin (*open symbols*) and with thapsigargin (2 mol/mol of Ca^{2+}-ATPase) (*closed symbols*). The *lines* show the best fit of the data using an equation for a first order decay process.

constant for the disappearance of this species at pH 6.0 and at pH 7.0, but the effect is much more marked in the presence of the co-solvent, both at pH 7.0 and 6.0. Me_2SO itself has either little effect (pH 7.0) or is mildly inhibitory (pH 6.0). Therefore the combined effect of thapsigargin and Me_2SO is synergistic;

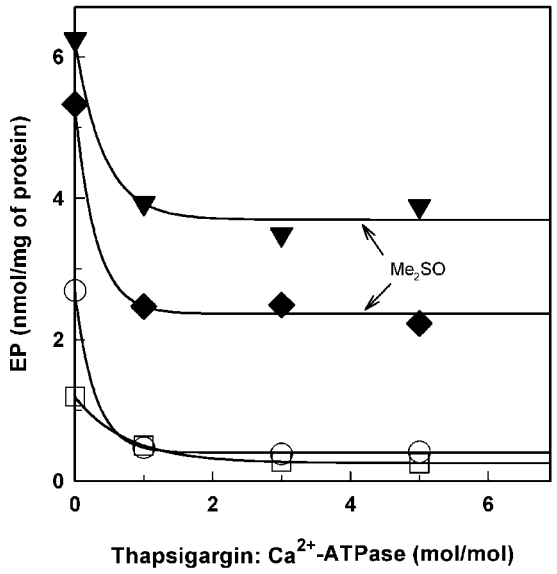


FIG. 4. **Effect of thapsigargin on E2P levels.** Increasing thapsigargin concentrations were added to SR vesicles (0.4 mg of protein/ml) incubated at 25 °C under the following conditions: 50 mM MES/Tris, pH 6.0, 20 mM $MgCl_2$, 1 mM EGTA, and 5 mM [$^{32}P_i$]P_i (○); 50 mM MES/Tris, pH 6.0, 20 mM $MgCl_2$, 30% (v/v) Me_2SO, 1 mM EGTA, and 2 mM [$^{32}P_i$]P_i (▼); 50 mM MOPS/TMAH, pH 7.0, 10 mM $MgCl_2$, 1 mM EGTA, and 2 mM [$^{32}P_i$]P_I (□); and 50 mM MOPS/TMAH, pH 7.0, 10 mM $MgCl_2$, 30% (v/v) Me_2SO, 1 mM EGTA, and 2 mM [$^{32}P_i$])P_i (♦).

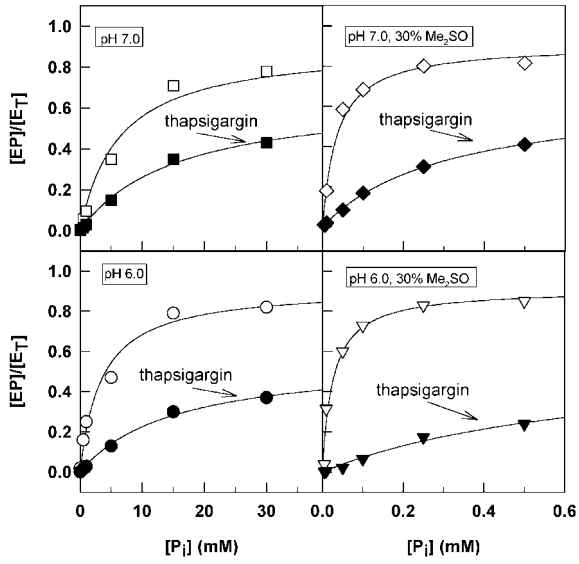


FIG. 5. **P_i concentration dependence of E2P formation.** SR vesicles (0.4 mg of protein/ml) were incubated with increasing concentrations of [$^{32}P_i$]P_i in 50 mM MOPS/TMAH, pH 7.0, 10 mM $MgCl_2$, and 1 mM EGTA (*upper left panel*); 50 mM MOPS/TMAH, pH 7.0, 10 mM $MgCl_2$, 30% (v/v) Me_2SO, and 1 mM EGTA (*upper right panel*); 50 mM MES/Tris, pH 6.0, 20 mM $MgCl_2$, and 1 mM EGTA (*lower left panel*); and 50 mM MES/Tris, pH 6.0, 20 mM $MgCl_2$, 30% (v/v) Me_2SO, and 1 mM EGTA (*lower right panel*) without thapsigargin (*open symbols*) or with thapsigargin (2 mol/mol of Ca^{2+}-ATPase) (*closed symbols*). The *curves* show the best fit to the equation $EP/E_{total} = [P_i]/(K_1K_2 + (K_2 + 1)[P_i])$. The EP_{max} values are given in Table I.

together the effect is much greater than either alone or an additive contribution of each.

Thapsigargin Inhibition of E2P Formed from P_i—Effect of increasing concentrations of thapsigargin on E2P levels from P_i is shown in Fig. 4. Maximal inhibition was reached at a 1:1 molar ratio with Ca^{2+}-ATPase. Under each of the conditions tested, in the presence and absence of Me_2SO, at pH 6.0 and pH 7.0, the inhibition by thapsigargin was only partial, in agreement with the findings of Sagara *et al.* (13). Molar ratios of up to 5:1 do not change this situation.

Effect of Thapsigargin and Me_2SO on K_1 and K_2 and Underlying Rate Constants—The P_i concentration dependences of E2P formation at pH 7.0 and 6.0 in the presence and absence of 30% (v/v) Me_2SO are shown in Fig. 5. EP_{max} and derived constants, K_1 and K_2, are shown in Table I. Note that K_1 is a dissociation constant equal to k_{-1}/k_1, and similarly K_2 is equal to k_{-2}/k_2. The preparation was the same as that used in Fig. 1 under "Experimental Procedures," where the FITC reactive Ca^{2+}-ATPase, and here taken to equal E_{total}, was 6.9 nmol/mg of protein.

Scrutiny of the K_1 and K_2 values reveals, first, that 30% (v/v) Me_2SO strongly decreases K_1 and has no effect on K_2, at both pH 6.0 and pH 7.0, indicating that the well known apparent affinity increase is a real one and entirely attributable to this change. In contrast, the main effect of thapsigargin is to increase K_2, in agreement with earlier findings (13). Together both effects remain, suggesting that the co-solvent targets the binding step and that thapsigargin targets the second covalent step. However, this is not entirely true as will be seen below.

Combining oxygen exchange and EP data allows evaluation of the four rate constants of Scheme 1, and the results are shown in Table I. Firstly, in the absence of thapsigargin and/or Me_2SO, the data show that k_{-2} is the slowest step in the reaction (12 and 4.6 s^{-1} at pH 7.0 and 6.0, respectively), and, because $K_2 = 0.1$, k_2 is 10-fold greater. The partition coefficient, P_c, then sets k_{-1} (11000 and 800 s^{-1} at pH 7.0 and 6.0, respectively). The large difference between the values at the two pH levels reflects the fact that the P_c is very close to 0 (virtually no reversal of bound P_i to phosphoenzyme before being released), and therefore small differences in P_c make for large differences in the rate constant. K_1 sets k_1, and it can be seen that productive binding is very much less than diffusion controlled binding, and this slow binding has been observed and remarked on before (3).

Analysis of the effects of thapsigargin and Me_2SO separately indicates that the increase in K_2 caused by thapsigargin is largely due to a 3–7-fold acceleration of k_{-2}. The forward rate constant k_2 is not affected or mildly inhibited. The large change in K_1 caused by Me_2SO alone is seen to be due to a very large inhibitory effect on k_{-1}. There are lesser inhibitory effects on k_2 and k_{-2}, to approximately equal extents because K_2 is not much changed.

Thapsigargin and Me_2SO together cause a large, 9-fold increase in k_{-2} at both pH values, without affecting k_2 much, when compared with the situation in the absence of these agents. Similarly, there is a large 17–32-fold inhibition of k_{-1} with little change in k_1. The selective inhibition of k_{-1} over k_2 is reflected in the increased P_c, the partitioning of P_i between release and covalent reaction. The basis for the accelerated oxygen exchange (note: total exchange = k_{-2} [EP]) is thus: (*a*) an inhibition of k_{-1} and therefore an increase in concentration of $E{\cdot}P_i$ and hence EP, (*b*) an acceleration of k_{-2}, and to a lesser extent (*c*) overcoming the inhibitory effect of either agent alone on k_2, which helps to ensure that EP remains fairly high in the face of a raised k_{-2}.

The finding that Me_2SO exerts a true affinity change for P_i agrees with the predictions of de Meis *et al.* (4), and the mechanism is due to a slowing of the "off" rate for P_i. Evidently, the co-solvent stabilizes phosphate-protein interactions. This causes an increase in the number of reversals of bound P_i, reflected in the increase in P_c, an effect noted by Champeil *et al.* (3), previously. Thapsigargin has little or no affect on P_i affinity in the absence or presence of Me_2SO.

To what extent does the value of E_{total} influence these con-

TABLE I
Kinetic parameters of P_i reaction with Ca^{2+}-ATPase

Conditions	EP_{max}[a]	$v_{exchange}$[b]	$EP_{exchange}$[c]	P_c[d]	K_1[f]	k_1[f]	k_1[f]	K_2[f]	k_2[f]	k_2[e]	k_{phos}[g]
	nmol·mg^{-1}	*nmol·min^{-1}·mg^{-1}*	*nmol·mg^{-1}*		*M*	*M^{-1}·s^{-1}*	*s^{-1}*		*s^{-1}*	*s^{-1}*	*s^{-1}*
pH 6.0											
−TG	6.4	580	2.1	0.06	4.2×10^{-2}	1.9×10^{4}	800	8.8×10^{-2}	46	4.6	13
+TG	3.8	780	0.4	0.11	3.5×10^{-2}	9.9×10^{3}	340	0.76	42	32	34
pH 6.0, 30% Me_2SO											
−TG	6.3	220	6.3	0.63	2.9×10^{-4}	1.2×10^{4}	3.6	9.9×10^{-2}	6.1	0.61	6
+TG	4.0	9.7×10^{3}	4.0	0.55	1.7×10^{-3}	2.9×10^{4}	48	0.71	57	41	62
pH 7.0											
−TG	≈6.3[h]	960	1.3	0.01	6.9×10^{-2}	1.7×10^{5}	1.1×10^{4}	0.10	120	12	28
+TG	4.2	1.2×10^{3}	0.5	0.07	4.7×10^{-2}	8.5×10^{4}	4.0×10^{3}	0.50	82	41	52
pH 7.0, 30% Me_2SO											
−TG	6.3	1.3 ± 10^{3}	6.3	0.30	3.8×10^{-4}	2.2×10^{5}	83	0.10	36	3.6	37
+TG	4.6	3.1×10^{4}	4.6	0.40	9.4×10^{-4}	3.6×10^{5}	340	0.49	226	110	284

[a] The maximum level of E2P formed by phosphorylation with [^{32}P]P_i at 25 °C under the conditions described in the legend to Fig. 5.
[b] Total rate of exchange ($v_{exchange}$) is calculated from $[(4 - 3P_c)/(4 - 4P_c)]4k_{av}[P_i]/[protein]$, where P_c is the partition coefficient, k_{av} is the rate constant for the average loss of ^{18}O for all four phosphate species, $[P_i]$ = 2 mM and [protein] = 0.4 mg of protein/ml. Conditions are as described in the legend to Fig. 3 (26).
[c] The E2P levels at the P_i concentration (2 mM) used in the assay for medium $P_i \leftrightarrow$ HOH oxygen exchange. Taken from the graphs in Fig. 5.
[d] The partition coefficient is determined from the equation $(4 - R_4)/3$, where R_4 is the ratio of the rate constant of $P^{18}O_4$ depletion to the rate constant of average ^{18}O depletion from all phosphate species (k_{av}). The first order disappearance of $P^{18}O_4$ is shown in Fig. 3, and that for the average loss was calculated from the same experiments (65).
[e] k_{-2} was determined from the relationship $v_{exchange} = k_{-2}$ [EP] (65) where [EP] is $EP_{exchange}$.
[f] E_{total} was determined in FITC labeling experiments and found to be 6.9 nmol/mg of protein (see "Experimental Procedures" and Fig. 1). K_1 and K_2 were obtained from fitting the data in Fig. 5. Rate constants k_1 and k_2 were calculated from $K_1 = k_{-1}/k_1$ and $K_2 = k_{-2}/k_2$, k_{-1} was determined from the relationship $P_c = k_2/(k_2 + k_{-1})$ (28).
[g] k_{phos}, the rate constant for formation of E2P, was determined from the $t_{0.5}$ of a simulated reaction where the P_i concentration was 10 mM and k_1, k_{-1}, k_2, and k_{-2} were as in this table.
[h] Fitting the data in Fig. 5 led to EP_{max} of 6.9 nmol/mg protein. This value is higher than obtained for the other curves, and because P_i has the lowest affinity under these conditions, it is subject to the most error. A value of 6.3 nmol/mg of protein was used in the calculations, which is an average of the other EP_{max} values. The experimental conditions are described in the legends to Figs. 3 and 5.

clusions? In particular, what if the FITC reaction overestimates the amount of active Ca^{2+}-ATPase? To answer this, the phosphorylation curves were also fitted with E_{total} equal to 6.33 nmol/mg of protein. This is slightly above the asymtotic EP_{max} value of 6.3 nmol/mg of protein (see Table I), which makes K_2 in the absence of thapsigargin extremely small, and the equilibrium of the covalent step well over to E2P. The calculated rate constants are shown in Table II. Firstly, it should be noted that k_{-2} is independent of E_{total}. Secondly, the rate constants in the presence of thapsigargin are hardly changed because EP_{max} under these conditions still remains significantly less than E_{total}. The principal change is in k_2 in the absence of thapsigargin, which is much larger, and this affects k_{-1} to the same extent. A much smaller K_2 profoundly increases K_1, which, when the effects on k_{-1} are assimilated, translates to k_1 remaining virtually unchanged. The effect of Me_2SO remains largely reflected in k_{-1}, *i.e.* a true affinity change. Thapsigargin alone now becomes strongly inhibitory on k_2 and k_{-1} but has little effect in the presence of the co-solvent. In the presence of both thapsigargin and Me_2SO, the activation of exchange remains the large decrease in k_{-1}, activation of k_{-2}, and, at pH 7.0, partial reversal of the individual inhibitory effects on k_2. Thus, the overall conclusions for the individual Me_2SO effect and the cause of activated exchange when in combination with thapsigargin are independent of E_{total}.

It should be mentioned that the reaction shown in Scheme 1 is a simplification. The unphosphorylated pump exists in an equilibrium of E1 and E2 forms, and low pH favors E2 and, hence, phosphorylation from P_i (1, 4). The species reacting with P_i is likely to be H_3·E2 (38). Also, high Mg^{2+} concentrations inhibit the phosphorylation reaction, particularly at neutral pH, probably by binding to the transport sites and converting the enzyme into an E1-like conformation (3, 38, 39). Luminal Ca^{2+}, increases the phosphorylation reaction by binding to *E*P, increasing the apparent affinity for P_i and largely overcoming Mg^{2+} inhibition (1, 3). Thus, the four rate constants may encompass more steps than that assigned to each (see also below).

The overall rate constant for the formation of *E*P from E and P_i (usually referred to experimentally as k_{obs}, but here referred to as k_{phos}) is a complex parameter dependent on all four rate constants. The computer-generated values for k_{phos} at 10 mM P_i are shown in the last columns of Tables I and II. Experimentally under similar conditions at pH 6.0 this rate constant has been found to be ~10 s^{-1} (3), and our values of 13 and 17 s^{-1} are similar to this. We find that 30% Me_2SO either has little effect or stimulates k_{phos} 2–3-fold, depending on whether E_{total} is taken as 6.9 or 6.33 nmol/mg of protein, respectively (Tables I and II). Larger increases (up to 7-fold) have been reported in the 0–20% concentration range (3), whereas at 40% Me_2SO there appears to be marked inhibition (4). It seems that Me_2SO alone stimulates k_{phos} at intermediate concentrations and inhibits at higher concentrations. Thus, our choice of an intermediate 30% may to some extent be obscuring other changes. The simulations show that k_{phos} is considerably accelerated in the presence of thapsigargin and Me_2SO (284–343 s^{-1}) under conditions of activated oxygen exchange (pH 7.0).

Implications for the Transport Sites and Energy Coupling—The second stage of the Ca^{2+}-ATPase catalytic cycle in the forward direction, namely that associated with the E2 forms and partial reactions of E2P hydrolysis and P_i release, is coupled with H^+ counter transport (40–42). During this stage of the cycle two or three H^+ ions are pumped out of the sarcoplasmic reticulum vesicles per Ca^{2+}-ATPase turnover. Details of H^+ translocation, the amino residues involved, associated conformational changes in the membrane helices, and the partial reactions at the catalytic site coupled to H^+ translocation are not well understood. In the homologous Na^+,K^+-ATPase, K^+ counter transport occurs during this part of the cycle (43, 44). K^+ binds tightly to the E2 state in the absence of Na^+ and is commonly referred to as being occluded. Phosphorylation from P_i promotes deocclusion to the other side of the membrane (physiologically to the cell exterior) to which the ions originally bound (43, 46, 47). Thus, one would predict that medium $P_i \leftrightarrow$ HOH oxygen exchange is associated with K^+ binding/dissocia-

TABLE II
Kinetic parameters of P_i reaction with Ca^{2+}-ATPase where E_{total} and EP_{max} are very close
Kinetic parameters are calculated as in Table I, except E_{total} was equal to 6.33 nmol/mg of protein.

Conditions	K_1	k_1	k_{-1}	K_2	k_2	k_{-2}	k_{phos}
	M	$M^{-1}\cdot s^{-1}$	s^{-1}		s^{-1}	s^{-1}	s^{-1}
pH 6.0							
−TG	0.52	2.2×10^4	1.1×10^4	6.2×10^{-3}	740	4.6	17
+TG	3.5×10^{-2}	1.1×10^4	388	0.67	48	32	37
pH 6.0, 30% Me_2SO							
−TG	3.0×10^{-3}	1.4×10^4	43	8.5×10^{-3}	73	0.61	36
+TG	1.8×10^{-3}	3.2×10^4	57	0.59	70	41	68
pH 7.0							
−TG	0.31	2.0×10^5	6.2×10^4	1.9×10^{-2}	630	12	31
+TG	6.2×10^{-2}	2.5×10^4	1.5×10^3	0.35	117	41	53
pH 7.0, 30% Me_2SO							
−TG	1.2×10^{-3}	2.4×10^5	290	2.9×10^{-2}	120	3.6	101
+TG	1.2×10^{-3}	3.8×10^5	470	0.35	310	110	343

tion and occlusion/deocclusion from the one side of the membrane (cell exterior). When applied to the Ca^{2+}-ATPase and assuming similar mechanisms, this means that the oxygen exchange is coupled to repeated protonation/deprotonation and occlusion/deocclusion events on the luminal side. Could this be happening in the thapsigargin/Ca^{2+}-ATPase complex?

Effect of Thapsigargin on Ca^{2+}-ATPase Stability—The effect of thapsigargin binding on the membrane helices was investigated by measuring its effect on denaturation of the Ca^{2+}-ATPase in detergent. In a Ca^{2+}-depleted medium, Ca^{2+}-ATPase is highly unstable when nonionic detergent substitutes for the natural phospholipids, presumably because there is loss of stabilizing interactions between helices in the detergent micelle (Ref. 37 and references therein). Evidently Ca^{2+} binding, which links membrane helices 4, 5, 6, and 8 (49), stabilizes the protein, at least in part, by holding the helices together. Denatured Ca^{2+}-ATPase in detergent can be measured by glutaraldehyde cross-linking (36, 37). The denatured protein cross-links much more readily than native solubilized monomer. The effect of thapsigargin on the stability of the solubilized monomer is shown in Fig. 6. With the high concentrations of glutaraldehyde employed in the assay, the membranous Ca^{2+}-ATPase (no detergent) cross-links to polymers that do not enter the gel (*lane 2*). This occurs irrespective of Ca^{2+}, ATP, or thapsigargin (data not shown). In detergent and in the presence of Ca^{2+}, the protein is monomeric and not cross-linked intermolecularly (*lane 3*). In the absence of free Ca^{2+}, the protein is readily cross-linked to polymers because of denaturation (*lane 4*). The addition of stoichiometric or higher amounts of thapsigargin prevented cross-linking and hence denaturation (*lanes 6–8*). These results are in agreement with other studies from our laboratory showing thapsigargin stabilization of Ca^{2+}-ATPase (16, 50) and with thapsigargin stabilization of tubular crystals (23). The "clamping" of membrane helices is also seen in the inhibition of passive Ca^{2+} efflux through the Ca^{2+}-ATPase from loaded vesicles by thapsigargin (51). It seems likely that thapsigargin locks the membrane helices in a manner that limits segmental or flexing movements.

Although side chain protonation and occlusion events at the transport sites may involve only small movements of the membrane helices, it appears unlikely, in view of the above findings, that these continue in an accelerated fashion with thapsigargin bound. One is tentatively led to conclude that the P_i reactions in the presence of thapsigargin are not associated with major transport site changes, and a mechanism such as in Scheme 2 can be put forward. Here medium $P_i \leftrightarrow$ HOH oxygen exchange and the associated reactions of P_i binding and phosphorylation only occur on intermediates with occluded H^+, a form stabilized by thapsigargin and Me_2SO (dotted box). Protonation of the transport sites from the medium side and occlusion occur on

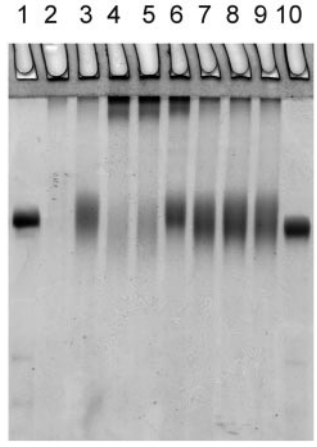
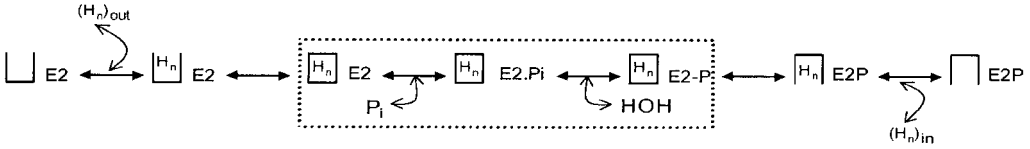


FIG. 6. **Effect of thapsigargin on the stability of Ca^{2+}-ATPase in detergent assessed by glutaraldehyde cross-linking.** SR vesicles (0.4 mg of protein/ml) were preincubated for 15 min at 25 °C in 50 mM MOPS/TMAH, pH 7.5, 10 mM $MgCl_2$, and 0.1 M sucrose with the additions given below. The glutaraldehyde cross-linking reaction (25 °C) was then initiated, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis. *Lanes 1* and *10*, no additions (control with no cross-linking; *lane 2*, no additions (control with cross-linking); *lane 3*, 0.5% Triton X-100 (w/v) and 0.1 mM $CaCl_2$; *lane 4*, 0.5% (w/v) Triton X-100 and 5 mM EGTA; *lanes 5–9*, 0.5% (w/v) Triton X-100, 5 mM EGTA, and increasing amounts of thaspigargin at 0.1, 0.5, 1, 2, and 3 mol/mol of Ca^{2+}-ATPase, respectively.

the unphosphorylated Ca^{2+}-ATPase, whereas these events from the luminal side take place on the phosphorylated form. One implication is that E2P species exist that are unreactive to water. In the related Na^+,K^+-ATPase, heterogeneous E2P species have been found that depend on cation identity, site occupancy, and whether the cations are occluded (52). A prediction of Scheme 2 is that transport site mutants that are unable to become protonated and occlude H^+ from the vesicle lumen would show retarded hydrolysis of E2P. In this regard, it is interesting that mutations Glu-309 to Gln and Glu-771 to Gln, both Ca^{2+}-binding residues, are able to be phosphorylated from P_i, indeed show increased apparent affinity for this substrate and exhibit reduced rates of dephosphorylation (53). The authors speculated that these glutamates participate in H^+ binding at luminal sites (53). It should be possible to test the validity of Scheme 2 with these mutants. According to the proposed mechanism, thapsigargin should stabilize the phosphoenzyme reactive to water and accelerate oxygen exchange. On the other hand if it also stabilizes species with luminally exposed transport sites with an active site unreactive to water, the exchange would not be accelerated. Another implication of Scheme 2 is that P_i binding and phosphorylation are not involved in H^+ occlusion *per se* but may act as a switch for



SCHEME 2

dictating the direction of proton access to the one side of the membrane or the other. The model receives support from a preliminary atomic structure of the Ca^{2+}-ATPase complexed with MgF_4 in a Ca^{2+}-depleted medium.[3] The latter compound mimics P_i and binds very tightly to the protein (54, 55), resulting in a form of the protein that could resemble E2P. Significantly, there is no obvious water-filled channel connected to the lumen in the atomic structure.

On the other hand, phosphorylation by P_i seems to produce large changes in the three cytosolic head domains. Fitting the atomic model of Ca^{2+}-bound Ca^{2+}-ATPase to an 8 Å resolution map obtained from two-dimensional tubular crystals formed in the absence of Ca^{2+} and presence of decavanadate and dansylthapsigargin suggests the gathering of the three cytoplasmic domains (49, 56). This is confirmed in the MgF_4 structure mentioned above. Mg/F-bound Ca^{2+}-ATPase has altered sulfydryl reactivity and is more heat-stable than control protein (57). Ca^{2+}-ATPase in the unphosphorylated Ca^{2+}-free form is rapidly digested by several proteases but is rendered almost completely resistant by P_i phosphorylation, Mg^{2+}/F^- treatment, orthovanadate, and decavanadate binding (58), again attesting to a compact E2P structure. It has been known for some time that P_i phosphorylation or vanadate binding is associated with a greatly increased fluorescence of bound 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (59, 60).

An alternative explanation for our results that may account for the apparent incompatibility between the protein exhibiting locked membrane helices and large cytoplasmic domain movements is that thapsigargin causes uncoupling of transport site and active site events. Thapsigargin may perturb the structure to permit P_i binding and phosphorylation without the usual changes in the protonation state of the transport sites. Both thapsigargin and Me_2SO alter the specificity of the active site in the E2 state, because they allow slow hydrolysis of ATP in the absence of Ca^{2+} (61, 62), normally a reaction that is dependent on Ca^{2+}. The solvent cannot be the sole cause of uncoupling because even in its absence the oxygen exchange catalyzed by the thapsigargin/Ca^{2+} complex is appreciable (Table I).

Thapsigargin binding enhances the attack of water on the phosphoenzyme. The structural changes induced by thapsigargin are rather slight as determined from analysis of tubular crystals but may include bending of the otherwise continuous M3/S3 helix (23). This helix is hydrogen bonded to the critical M6/M7 loop as well as to a stirrup-like helix leading directly to the β-strand with Asp-351, which is phosphorylated (49). The M6/M7 loop wraps around the top section of the central extended pillar helix M5. Mutations to the loop inhibit P_i phosphorylation (63), whereas several in the pillar helix activate E2P hydrolysis (64). In particular, mutating either Ile-743 or Tyr-754 to alanine accelerates dephosphorylation ~20-fold. Evidently the M5 mutations, like thapsigargin, are effecting changes at the catalytic site thereby promoting the attack of water on the phosphoryl group, whereas those in the loop do the opposite. These effects may be achieved in part through Thr-353 in the conserved phosphorylation loop because it could be acting as the general acid/base for the activation of the water molecule (48). The hydroxyl group is essential for P_i phosphorylation, whereas mutating Thr-353 to serine stimulates E2P hydrolysis up to 22-fold (48). Thus, small changes in the disposition of Thr-353 may produce profound changes in this reaction.

In conclusion, thapsigargin and Me_2SO promote an accelerated phosphorylation and dephosphorylation cycle in the E2 state caused mainly by activation of *E*P hydrolysis and inhibition of P_i release. Thapsigargin stabilizes the membrane helices, and these reactions at the catalytic site may be occurring with little membrane helix movement, possibly with the transport sites in a locked, protonated state.

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Thapsigargin and Dimethyl Sulfoxide Activate Medium P_i <=> HOH Oxygen Exchange Catalyzed by Sarcoplasmic Reticulum Ca^{2+}-ATPase

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