Characterization of the Inhibition of Intracellular Ca\textsuperscript{2+} Transport ATPases by Thapsigargin*

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The effects of thapsigargin (TG), a specific inhibitor of intracellular Ca\textsuperscript{2+}-ATPases, were studied on vesicular fragments of sarcoplasmic reticulum (SR) membranes. Inhibition of Ca\textsuperscript{2+} transport and ATPase activity was observed following stoichiometric titration of the membrane-bound enzyme with TG. When Ca\textsuperscript{2+} binding to the enzyme was measured in the absence of ATP, or when one cycle of Ca\textsuperscript{2+}-dependent enzyme phosphorylation by ATP was measured under conditions preventing turnover, protection against TG by Ca\textsuperscript{2+} was observed. The protection by Ca\textsuperscript{2+} disappeared if the phosphoenzyme was allowed to undergo turnover, indicating that a state reactive to TG is produced during enzyme turnover, whereby a dead-end complex with TG is formed.

Enzyme phosphorylation with P\textsubscript{i}, ATP synthesis, and Ca\textsuperscript{2+} efflux by the ATPase in its reverse cycling were also inhibited by TG. However, under selected conditions (millimolar Ca\textsuperscript{2+} in the lumen of the vesicles, and 20% dimethyl sulfoxide in the medium) TG permitted very low rates of enzyme phosphorylation with P\textsubscript{i} and ATP synthesis in the presence of ADP. It is concluded that the mechanism of ATPase inhibition by TG involves mutual exclusion of TG and high-affinity binding of external Ca\textsuperscript{2+}, as well as strong (but not total) inhibition of other partial reactions of the ATPase cycle. TG reacts selectively with the state acquired by enzyme exposure to EGTA, or by utilization of ATP and consequent displacement of bound Ca\textsuperscript{2+} during catalytic turnover.

Thapsigargin (TG), a plant-derived sesquiterpene lactone (Christensen et al., 1981) with tumor-promoting properties, interferes with control of the intracellular Ca\textsuperscript{2+} concentration through inhibition of sequestration mechanisms (Thastrup et al., 1990). TG inhibits Ca\textsuperscript{2+} transport by SR vesicles isolated from skeletal muscle (Sagara and Inesi, 1991; Kijima et al., 1991), as well as all isoforms of the endo- and sarcoplasmic reticulum Ca\textsuperscript{2+} (SERCA) pumps (Campbell et al., 1991; Lytton et al., 1991). TG is a specific inhibitor of intracellular Ca\textsuperscript{2+} pumps inasmuch as neither the plasma membrane Ca\textsuperscript{2+} pump, nor other cation pumps are affected (Lytton et al., 1991). The importance of TG as an experimental tool lies in its ability to interfere specifically with intracellular Ca\textsuperscript{2+} trapping systems, and the possibility to test the effect of this interference on the large number of Ca\textsuperscript{2+}-dependent intracellular functions. TG is also an important tool for the molecular characterization of the coupling mechanism of catalysis and transport in the Ca\textsuperscript{2+}-ATPase.

Owing to natural abundance and high density of pump units, vesicular fragments of SR obtained from skeletal muscle constitute an ideal system for mechanistic studies of TG inhibition. Experimentation with this system revealed that TG binds to the SR ATPase stoichiometrically with very high affinity, producing inhibition of Ca\textsuperscript{2+} binding in the absence of ATP and of catalytic activity when ATP is added (Sagara and Inesi, 1991). Furthermore, TG reverses the intrinsic fluorescence signal produced by Ca\textsuperscript{2+} binding to the ATPase, and allows formation of bidimensional crystalline arrays of the ATPase even in the presence of Ca\textsuperscript{2+} which normally interferes with crystallization (Sagara et al., 1992).

We are here describing a series of experiments on the characterization of the TG inhibition, including its antagonism with Ca\textsuperscript{2+}, its preferential interaction with a specific state of the ATPase, and its inhibition of various partial reactions of the catalytic and transport cycle. With these experiments we tested overall ATPase and transport activities, as well as the following partial reactions.

\begin{equation}
E + 2Ca^{2+} \rightleftharpoons ECa^{2+} \\
ECa^{2+} + ATP \rightleftharpoons E-PCa^{2+} + ADP \\
E-PCa^{2+} \rightleftharpoons E-P + 2 Ca^{2+} \\
E-P \rightleftharpoons E + P_i
\end{equation}

These reactions, which were selected for their experimental accessibility, are likely to include conformational transitions that may account for vectorial translocation of bound Ca\textsuperscript{2+} and certain kinetic features of the system (de Meis and Vianna, 1979; Inesi et al., 1980; Petithory and Jencks, 1986). Conformational transitions are also suggested by our experiments with TG, and will be considered in the discussion of the experimental results.

MATERIALS AND METHODS

Vesicular fragments of longitudinal SR were obtained from rabbit skeletal muscle according to Eletr and Inesi (1972). Thapsigargin was purchased from LC Services Corp., Woburn, MA. All other chemicals were purchased from Sigma. Cr(H\textsubscript{2}O\textsubscript{4})\textsubscript{2}ATP bidentate was prepared by the method of Dunaway-Mariano and Cleland (1980).

Protein concentration was measured by the method of Lowry et al. (1951), standardized with bovine serum albumin.

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Protein concentration was measured by the method of Lowry et al. (1951), standardized with bovine serum albumin.
ATP-dependent Ca\(^{2+}\) uptake by SR vesicles was followed by measuring fluxes of radioactive \(^{45}\)Ca tracer. For this purpose, the vesicles were incubated in reaction mixtures specified in the Figure legends, and serial samples were collected on 0.45-μm Millipore filters and washed three times with 5 ml of cold 2 mM LaCl\(_3\) plus 10 mM MOPS, pH 7. After washing, the filters were dissolved in 0.5 ml of dimethyl formamide, and processed for scintillation counting.

ATPase activity was followed by measuring production of P\(_i\), according to Lin and Morales (1977), and velocity was estimated from the slope of several time points on a straight line.

Calcium binding by the SR ATPase in the absence of ATP was determined by placing SR vesicles (100 μg of protein) on a 0.65-μm Millipore filter and perfusing them with relevant reaction mixtures (see Figure legends). The perfusion time was electronically controlled with the aid of a Bio-Logic rapid filtration apparatus. The filters were then collected for determination of radioactive (\(^{45}\)Ca) tracer associated with the vesicles.

Calcium occlusion by the ATPase in the presence of CrATP was obtained in a reaction mixture including 100 mM MOPS, pH 7.0, 80 mM KCl, 0.1 mM \(^{45}\)CaCl\(_2\), 0.2 mM EGTA, 2 μM A23187, and 0.2 mg of SR protein/ml. The reaction was started by the addition of 0.5 mM CrATP at 25 °C, and 0.25-ml samples were collected at serial times. These samples were placed in 2 mM LaCl\(_3\) plus 10 mM MOPS, pH 7, and, after 2 min, filtered through 0.45-μm Millipore filters. The filters were washed three times with 4 ml of 2 mM LaCl\(_3\) plus 10 mM MOPS, pH 7, and processed for determination of \(^{45}\)Ca by scintillation counting. Blanks obtained in the absence of CrATP were subtracted from the values obtained with CrATP.

Calcium occlusion time intermediate was obtained either by utilization of \([\gamma-\text{\textsuperscript{32}P}]\text{ATP in the presence of Ca}^{2+}\), or of \([\text{\textsuperscript{32}P}]\text{Pi}\), in the absence of Ca\(^{2+}\) (see Figure legends for reaction conditions). Incubations were terminated by the addition of a volume of 1 mM PCA plus 4 mM P\(_i\) equal to the reaction mixtures. The quenched samples, containing 0.5–1.0 mg of protein, were sedimented and washed three times with 0.25 M PCA plus 4 mM P\(_i\). The final sediment was dissolved for determination of radioactive phosphorus and protein.

Alternatively, small aliquots (microgram range) of quenched protein were placed on 0.45-μm Millipore filters, washed extensively with cold 0.1 N HCl and cold H\(_2\)O, and processed for determination of radioactivity on the filters.

For studies of the reversal of the Ca\(^{2+}\) pump, SR vesicles (35–45 μg of protein/ml) were preloaded with Ca\(^{2+}\) in a reaction mixture containing 50 mM MOPS-Tris, 10 mM MgCl\(_2\), 20 mM P\(_i\)-Tris, 0.3 mM CaCl\(_2\) (in the presence or in the absence of radioactive \(^{45}\)Ca tracer), and 5 mM ATP. Following 40 min incubation at 37 °C, 5-ml samples were centrifuged at 4°C, and the supernatant was reconstituted with 0.45 ml of ice-cold water. 50-μl aliquots of this suspension were then diluted in 1.5 (or more) ml of efflux medium containing 50 mM MOPS-Tris, pH 7.0, 10 mM MgCl\(_2\), 4.0 mM P\(_i\)-Tris (in the presence or in the absence of radioactive \(^{45}\)P tracer), 2 mM EGTA, 10 mM glucose, and 20 μg of hexokinase/ml, in the presence or in the absence of 0.2 mM ADP at 37 °C. Serial samples were then filtered and washed with LaCl\(_3\) for determination of \(^{45}\)Ca remaining associated with the vesicles, or quenched with an equal volume of 1 M PCA plus 4 mM P\(_i\), for determination of ATP synthesis (de Meis, 1968).

**RESULTS**

**Apparent Irreversibility of ATPase Inhibition by Thapsigargin**—TG reacts with the SR ATPase with very high affinity, resulting in enzyme inhibition which is proportional to stoichiometric titration of the ATPase with TG (Sagara and Inesi, 1991). We found that a tight stoichiometric ratio (1:1) of added TG and inactivated ATPase is obtained when aliquots of TG dissolved in dimethyl sulfoxide are added directly to an aqueous suspension of SR vesicles, avoiding preincubation dilution of TG in aqueous media in the absence of SR vesicles.

In the experiment shown in Fig. 1 we added to a concentrated suspension of SR vesicles an amount of TG stoichiometrically equivalent to approximately half of the Ca\(^{2+}\)-ATPase. The resulting partial inhibition of Ca\(^{2+}\) transport activity corresponds to stoichiometric titration of the ATPase with TG. Under these conditions the concentration of free TG in the medium is expected to be near its \(K_a\) and, therefore, the extent of protein titration should be very sensitive to changes in the concentration of free TG. Nevertheless, a 10,000-fold dilution of the inhibited protein suspension with medium containing no TG, resulted in no reversal of inhibition within a period of 2 h following dilution. This indicates that dissociation of the TG-enzyme complex is very slow, consistent with the high affinity of the inhibitor for the enzyme. On the other hand, if the ATPase treated with TG was digested extensively with trypsin or denatured with urea, TG was recovered in the washing medium of the denatured preparation, as demonstrated by inhibition of a new batch of SR vesicles. No TG was recovered by washing TG-treated vesicles with non-denaturing media (data not shown).

**Inhibition of Steady State Activity in the Presence of Various Concentrations of ATP and Ca\(^{2+}\)**—When steady state ATPase velocity was measured in the presence of various ATP concentrations, we found that TG inhibition was independent of the ATP concentration (Fig. 2A). By comparison, a clearly competitive behavior (Fig. 2A) is displayed by cyclopiazonic acid which is another inhibitor of the SR ATPase (Seidler et al., 1989). This suggests that the TG inhibition is not related to the concentration of ATP present in the medium. We also found that, under steady state conditions, the ATPase inhibition by TG is not reversed by addition of higher Ca\(^{2+}\) concentrations (Fig. 2B). Nevertheless, in pre-steady state studies of partial ATPase reactions we noted that the time course of enzyme inactivation by TG was dependent on the order of reagent additions to the reaction mixture.

**Inhibition of Ca\(^{2+}\) Binding in the Absence of ATP**—High affinity binding of external Ca\(^{2+}\) may be considered the first partial reaction of the ATPase catalytic cycle (see reaction sequence in the Introduction), as the bound Ca\(^{2+}\) produces enzyme activation and in turn undergoes vectorial displacement upon enzyme phosphorylation by ATP. In the absence of ATP, Ca\(^{2+}\) binding to the enzyme can be measured under equilibrium conditions as a self-limited partial reaction which we found previously to be inhibited by TG (Sagara and Inesi, 1989).
Fig. 2. ATPase velocity as a function of ATP and Ca\(^{2+}\) concentrations: inhibition by TG. The reaction mixture contained 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 10 \(\mu\)g of SR protein/ml, and 5 \(\mu\)M A23187. The reaction mixture for A contained also 0.20 mM EGTA, 0.2 mM CaCl\(_2\), 2 mM phosphoenolpyruvate, 30 \(\mu\)g of pyruvate kinase/ml, and ATP as specified in the abscissa. The reaction mixture for B contained 0.1 mM EGTA, various concentrations of CaCl\(_2\), to yield the free Ca\(^{2+}\) concentrations specified in the abscissa, and 2.5 mM ATP. When indicated, TG was 50 (\(\Delta\)) and 25 nM (\(\triangledown\)).

Fig. 3. Time course of TG inhibition of Ca\(^{2+}\) binding by SR ATPase: protection by Ca\(^{2+}\). SR vesicles were suspended (50 \(\mu\)g of protein/ml) in a medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), and various concentrations of \(^{45}\)CaCl\(_2\) as indicated in the Figure. Following 5 min preincubation at 25 °C, TG (10 nmol/mg SR protein) was added, and 2-ml samples were filtered at variable times (indicated in the abscissa) through 0.65-\(\mu\)m Millipore filters placed on a rapid filtration Bio-logic apparatus. The vesicles remaining on the filters were then perfused for 0.6 s with a medium 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), and 5 \(\mu\)M \[^{45}\text{Ca}\]CaCl\(_2\). The filters were then collected without any washing for determination of radioactive calcium bound to the vesicles. Blanks were obtained with filters perfused in the absence of vesicles.

**Inhibition of Enzyme Phosphorylation with ATP**—Addition of ATP to SR vesicles preincubated with Ca\(^{2+}\) leads to formation of a phosphorylated enzyme intermediate by transfer of the ATP terminal phosphate to the ATPase (Yamamoto and Tonomura, 1968). If this reaction is carried out in ice for a short time (e.g. 1 s) and in the absence of ADP, nearly stoichiometric phosphorylation of the enzyme is obtained since the rate of phosphoenzyme formation is faster (Froehlich and Taylor, 1975) and less temperature dependent than its hydrolytic cleavage.

In analogy to the previous experiment on Ca\(^{2+}\) binding, we then incubated SR vesicles at 15 °C with TG for increasing time intervals in the absence (i.e. presence of EGTA) or in the presence of Ca\(^{2+}\), and then added ATP (plus Ca\(^{2+}\) to ensure its presence during phosphorylation) for 1 s at ice temperature. Even in this case we found that the inhibition was much more pronounced when SR vesicles were exposed to TG in the absence of Ca\(^{2+}\), while much less inhibition was obtained when 10 or 50 \(\mu\)M Ca\(^{2+}\) was present during the incubation with TG (Fig. 4A).

The experiments shown in Figs. 3 and 4A indicate that Ca\(^{2+}\) protects the ATPase from inactivation by TG. We also found (Fig. 4, B and C) that protection by Ca\(^{2+}\) is least effective at high temperature (37 °C) and low pH, pH 6.0, and most effective at low temperature (2 °C) and high pH, pH 8.0. It is likely that these differences are related to the temperature and pH dependence of ATPase conformational changes determining the sensitivity of the enzyme to inhibition by TG.

It is of interest that when ATP is added with TG in the presence of Ca\(^{2+}\), and samples are taken at serial time intervals for determination of phosphoenzyme, inactivation occurs over a period of 30 s (Fig. 5), indicating that catalytic turnover produces an enzyme species which is receptive to TG thereby yielding a dead end complex. This explains the apparent

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1991). We now find that preincubation with Ca\(^{2+}\) has a protective effect against TG.

In the experiment shown in Fig. 3, we incubated SR vesicles with TG in the presence of various concentrations of nonradioactive Ca\(^{2+}\) for increasing time intervals, before placing them on a rapid filtration apparatus for removal of the incubation medium. The vesicles remaining on the filters were then perfused for 0.6 s with a medium containing radioactive Ca\(^{2+}\) (isotopic calcium exchange is quite rapid under these conditions), to test their residual Ca\(^{2+}\) binding activity. We found that inhibition of Ca\(^{2+}\) binding was inversely proportional to the Ca\(^{2+}\) concentration present during the incubation with TG (Fig. 3). Therefore, in apparent contrast with inhibition of steady state ATPase activity, equilibrium measurements of Ca\(^{2+}\) binding in the absence of ATP show protection of the ATPase by Ca\(^{2+}\) against TG.
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**Fig. 4.** Time course of TG inhibition of ATPase phosphorylation with ATP; protection by Ca\(^{2+}\). A, SR vesicles were suspended (0.5 mg/ml) in a medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), and 50 \(\mu\)M CaCl\(_2\), in the absence (•) or in the presence (△) of TG (10 nmol/mg SR protein), at 4 °C. The reaction was started by the addition of 100 \(\mu\)M \(\gamma\)-\(^{32}\)P]ATP and quenched at serial times with an equal volume of 1 M PCA plus 5 mM P\(_2\), and the samples were processed for determination of radioactive phosphoenzyme.

**Fig. 5.** Time course of ATPase inhibition by TG during enzyme turnover. SR vesicles were suspended (0.5 mg/ml) in a buffer containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), and 50 \(\mu\)M CaCl\(_2\), in the absence (○) or in the presence (△) of TG (10 nmol/mg SR protein), at 4 °C. The phosphoenzyme levels obtained in the absence of TG (in the range of 2–3 nmol/mg of protein) were considered to be 100%.

Discrepancy between Fig. 5 showing that Ca\(^{2+}\) does not protect steady state enzyme activity from TG, and Fig. 4 showing protection under conditions preventing enzyme turnover. Particularly cogent is the comparison between Fig. 5 and the upper curve of Fig. 4B, both obtained at 4 °C and in the presence of 50 \(\mu\)M Ca\(^{2+}\), the only difference being the addition of ATP with TG in the experiment shown in Fig. 5.

Occlusion of Bound Ca\(^{2+}\) by CrATP—ATP utilization by the SR ATPase produces occlusion of bound Ca\(^{2+}\) before its release in the lumen of the vesicles (for review, see Glynn and Karlish (1990)). The occluded state can be obtained in a stable form by reacting the ATPase with CrATP in the presence of Ca\(^{2+}\) (Serperus et al., 1982; Vilsen and Andersen, 1987). Dissociation of radioactive occluded Ca\(^{2+}\) from the enzyme-CrATP complex is obtained upon addition of EGTA (Fig. 6) or excess nonradioactive calcium isotope (not shown). Such a dissociation is very slow, and includes two distinct kinetic components likely related to sequential dissociation of the two calcium ions bound sequentially to each mole of ATPase (Inesi et al., 1980; Dupont, 1982; Inesi, 1987). We found that TG prevents Ca\(^{2+}\) occlusion if added to the SR ATPase before CrATP (not shown). On the other hand, addition of TG to the preformed enzyme-CrATP complex is followed by dissociation of occluded Ca\(^{2+}\) with kinetics quite similar to those observed following addition of EGTA (Fig. 6). This suggests that in all cases the decay kinetics of occluded state levels reflect directly the rates of Ca\(^{2+}\) dissociation. Therefore the role of TG (in analogy of that of EGTA) is simply that of preventing Ca\(^{2+}\)-exchange following spontaneous dissociation of the occluded Ca\(^{2+}\). While EGTA exerts this effect by chelating free Ca\(^{2+}\) in the medium, TG prevents

\[ \text{Phosphorylation reaction was quenched after 1 s with an equal volume of 1 M PCA plus 5 mM P}_2\text{, and processed for determination of radioactive phosphoenzyme.} \]

\[ \text{BD, as A with 50 \(\mu\)M Ca\(^{2+}\), but incubated at various temperatures with TG. C, as in A with 50 \(\mu\)M Ca\(^{2+}\), but for the different pH which was maintained with a 20 mM BisTris-Tris buffer. For A-C control runs were conducted in the absence of TG. The phosphoenzyme levels obtained in the absence of TG (in the range of 2–3 nmol/mg of protein) were considered to be 100.} \]
the Pi reaction can occur either as
empty) vesicles are used (de Meis, 1988; Beil et al., 1988). As long as Ca\(^{2+}\) concentration in the medium is very low (0.1 mM), the concentration dependence of this reaction is lower if loaded (rather than empty) vesicles are used. The enzyme is phosphorylated through a preliminary incubation, as long as the Ca\(^{2+}\) concentration in the medium outside the vesicles is lowered below 0.1 mM. After 2 min the samples were filtered through 0.45-μm Millipore filters. The filters were rinsed 3 times with 10 mM MOPS, pH 7.0, and collected for determination of radioactive calcium.

Further binding by virtue of its own interaction with the enzyme. Since CrATP occupies the catalytic site as a stable complex either in the presence or in the absence of bound Ca\(^{2+}\) (Chen et al., 1991), the effect of TG cannot be attributed to TG binding within the catalytic and/or nucleotide-binding domain which is occupied by CrATP. These experiments also demonstrate that TG cannot affect the enzyme as long as Ca\(^{2+}\) is bound to it in its high affinity state.

Inhibition of Enzyme Phosphorylation with P\(_i\)—The Ca\(^{2+}\)-ATPase of SR can be phosphorylated with P\(_i\), instead of ATP provided that the enzyme is deprived of Ca\(^{2+}\) by the addition of EGTA (Masuda and de Meis, 1973; de Meis and Masuda, 1974). This reaction can be measured under equilibrium conditions independent of enzyme turnover, and can be considered the reversal of phosphoenzyme hydrolytic cleavage (see reaction sequence in the Introduction). As observed in equilibrium measurements of Ca\(^{2+}\) binding (Sagara and Inesi, 1989), degradation experiments (Sagara and Inesi, 1989) and 4.0 (a) nmol of TG/mg of protein. The reaction was allowed to proceed for 15 min at 25 °C before quenching with an equal volume of 1 M PCA plus 4 mM P\(_i\). The samples were then processed for determination of radioactive phosphoenzyme.

![Fig. 7. Enzyme phosphorylation by P\(_i\), as a function of P\(_i\) concentrations: inhibition by TG. SR vesicles were suspended (0.5 mg of SR protein/ml in a medium containing 50 mM MES-Tris, pH 6.2, and 2 mM EGTA. The medium contained 10 mM MgCl\(_2\), various concentrations (abscissa) of [\(^{32}\)P]P\(_i\) and 0 (□), 2.5 (▲), 3.5 (■), and 4.0 (▲) nmol of TG/mg of protein. The reaction was allowed to proceed for 15 min at 25 °C before quenching with an equal volume of 1 M PCA plus 4 mM P\(_i\). The samples were then processed for determination of radioactive phosphoenzyme.](image-url)

\[ *E + P_i \xrightleftharpoons{K_1} *E.P_i \xrightleftharpoons{K_2} *E-P \]  

or as

\[ *E \text{Ca}_{2+} + P_i \xrightleftharpoons{K_1} *E \text{Ca}_{2+}.P_i \xrightleftharpoons{K_2} *E \text{Ca}_{2+}.P \]  

with somewhat different characteristics (Beil et al., 1977; Chaloup et al., 1979). The denotation *E, as opposed to simple E, was introduced by de Meis and Vianna (1979) to distinguish the enzyme state which is promoted by the absence of Ca\(^{2+}\) in the high affinity outward oriented sites of the ATPase.

In the experiment shown in Figs. 7 and 9, we obtained equilibrium levels of 2.3 and 1.9 nmol of E-P/mg of protein with empty and loaded vesicles, respectively. Considering that the catalytic site stoichiometry was 5 nmol/mg protein, the E-P level suggest a K\(_p\) value near 1 in both cases.

As for the effect of TG, we found that the E-P level was
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Fig. 9. Enzyme phosphorylation with P, in loaded vesicles: effects of Ca^{++}_{out} and TG. SR vesicles preloaded with Ca^{++} as explained under "Materials and Methods," were suspended (12 μg of protein/ml) in a medium containing 50 mM MOPS-Tris, pH 7.0, 10 mM MgCl_2, 4 mM [35]P]Pi, either 2.0 mM EGTA (●) or 35 μM CaCl_2 (▲), and variable TG. 0.4-ml samples were quenched with an equal volume of 1 M PCA plus 4 mM P, following 1-min incubation at 37 °C, filtered, and washed for determination of [35]P]E-P.

If the Ca^{++} concentration in the medium is raised above micromolar, the P, reaction is inhibited (Masuda and de Meis, 1973), since the enzyme acquires different characteristics as a consequence of Ca^{++} binding to the high affinity sites on the outer surface of the vesicles, as in

\[ *E \rightleftharpoons E \rightarrow Ca^{++}_{out} \text{ ATP} \rightarrow E-PCa_{out} \]

where *E and E are two distinct states permitting enzyme phosphorylation only by P, or ATP, respectively. It is of interest that, while in the absence of TG the P, reaction is totally inhibited by 35 μM Ca^{++} (Fig. 9), addition of TG interferes with the Ca^{++} inhibition and brings back E-P to the same level observed with TG in the absence of Ca^{++}. This is due to TG inhibition of high affinity Ca^{++} binding and retention of the *E state by the enzyme. Consequently, the P, reaction is allowed to occur within the limits imposed by the K_c in Equations 2 and 3. It is apparent that this reduction is somewhat greater for E-P, than for ECA_{pre}-P.

If the Ca^{++} concentration in the medium is raised above 0.1 μM, when TG is added to match the enzyme stoichiometry (Figs. 7 and 9). Considering that the P, concentration dependence is not changed by TG, and that saturating P, was used, we conclude that TG lowers the levels of phosphoenzyme by reducing the equilibrium constant for the phosphorylation reaction (K_c in Equations 2 and 3). It is apparent that this reduction is somewhat greater for E-P, than for ECA_{pre}-P.

Inhibition of ATP Synthesis and Coupled Ca^{++} Efflux—It was originally discovered in Hasselbach's laboratory (Barogtie et al., 1971) that if SR vesicles preloaded with Ca^{++} in the presence of ATP and oxalate are diluted in a medium containing P, ADP, and low Ca^{++} (i.e. EGTA), a complete reversal of the pump is obtained, beginning with enzyme phosphorylation by P, and resulting in ATP synthesis and coupled Ca^{++} efflux. In our experiments we used vesicles preloaded in the presence of ATP and P, (instead of oxalate). In this case, owing to a higher solubility of the calcium-phosphate complex in the lumen of the vesicles, a higher rate of Ca^{++} efflux is observed upon dilution (Sande-Lemos and de Meis, 1988). A significant efflux component is observed even when the dilution medium does not contain ATP. The efflux increment obtained upon addition of ADP is then stoichiometrically (2:1 under favorable conditions) related to ATP synthesis.

When we tested the effect of TG on this system, we found that the ADP-independent efflux, which is an uncoupled function of a slow ATPase channel normally involved in coupled transport (Inesi and de Meis, 1989), was not inhibited by TG. On the other hand, the ADP-dependent increment of Ca^{++} efflux, as well as the coupled ATP synthesis were inhibited by stoichiometric titration of the enzyme with TG (Fig.
10, B and C). It is then apparent that TG inhibits the overall catalytic and coupled transport cycling of the SR ATPase, in both the forward and reverse directions. It can be shown, however, that under the conditions of the experiment illustrated in Fig. 9, the very small amounts of phosphoenzyme formed with P, in the presence of TG can be utilized to form ATP upon addition of ADP (Fig. 11).

**DISCUSSION**

With the experiments reported above we confirmed that TG interacts stoichiometrically with the SR ATPase producing an apparently irreversible inactivation. We also found that the presence of specific ATPase ligands and substrates has a profound influence on the effect of TG, indicating that TG forms a stable inhibitory complex only with a specific state of the ATPase.

As TG inhibits Ca\(^{2+}\) binding to the ATPase in the absence of ATP, this reaction can be studied under equilibrium conditions and in the absence of catalytic turnover. It is then found that Ca\(^{2+}\), if added to the SR vesicles before TG, has a protective effect (Figs. 3 and 4). This could be explained by the following reaction sequence:

\[
\text{TG} \cdot \text{E} \xrightarrow{\text{TG}} \text{E} \xrightarrow{2\text{Ca}^{2+}} \text{ECa}_2
\]

where occupancy of the Ca\(^{2+}\) and TG sites on the enzyme is mutually exclusive, and TG reacts only with *E. Owing to the very high association constant of Ca\(^{2+}\) for the enzyme, most of the enzyme is in the ECa\(_2\) form in the presence of micromolar Ca\(^{2+}\). Therefore, even though the TG affinity for the enzyme is seemingly stronger than that of Ca\(^{2+}\), the rate of TG - E formation is limited by the concentration of E which is very low if Ca\(^{2+}\) is added before TG. It is also possible that a significant concentration of the species TG - E - Ca\(_2\) is also formed, which then undergoes transformation to TG - *E and 2 Ca\(^{2+}\) at very low rates.

It is of interest that the protective effect of Ca\(^{2+}\) is less prominent at low temperature and at high pH (Fig. 4), since under these conditions the equilibrium between the two interconverting states *E and E appears to be shifted in favor of *E, as revealed by a higher reactivity to P, (Masuda and de Meis, 1973).

If TG is added to SR vesicles in the presence of Ca\(^{2+}\) concentrations delaying enzyme inactivation by TG in the absence of ATP (Figs. 4 and 5), a much faster enzyme inactivation is observed upon addition of ATP (Fig. 6). This inactivation is due to production of a reactive state as a consequence of ATP utilization, and trapping of E of the enzyme in a dead end complex. The catalytic and transport cycle of the SR ATPase may be represented as follows.

### Scheme 6

Scheme 6, which is derived from de Meis and Vianna (1979), was augmented by branched pathways for the phosphorylation reactions with ATP or P, to allow external Ca\(^{2+}\) to bind before or after ATP (Petithory and Jencks, 1986), and internal Ca\(^{2+}\) to bind before or after P, (Chaloub et al., 1979). For our present purpose, it was not necessary to include steps allowing for sequential Ca\(^{2+}\) binding (Inesi, 1987) or Ca\(^{2+}\) occlusion by enzyme phosphorylation (Glynn and Karlish, 1990). Whether the phosphorylated enzyme resides in two different conformations corresponding to E and *E, or prevalently in another conformation allowing occlusion of bound Ca\(^{2+}\), is a matter of controversy (Pickart and Jencks, 1982). At any rate, Scheme 6 provides an intuitive appreciation of how the enzyme would reside mostly in the E - Ca\(_2\) form in the presence of external Ca\(^{2+}\). Upon addition of ATP, the enzyme would then distribute itself in the various states, including *E which is reactive to TG, thereby permitting accumulation of the stable inhibitory complex TG - *E. The experiments described above indicate that *E (as opposed to E) is characterized by reactivity to P, and TG, relative stability in the absence of Ca\(^{2+}\) at low pH and high temperature, and relative instability in the presence of Ca\(^{2+}\) at high pH and low temperature. *E and TG - *E are distinguished from E by the low level of intrinsic fluorescence, and by their ability to form bidimensional crystalline arrays upon addition of decavanadate (Sagara et al.,...
As opposed to *E, however, TG·*E cannot bind external Ca²⁺ and, therefore, is unable to utilize ATP (Figs. 4 and 5). We found that TG inhibits also enzyme phosphorylation by Pₐ (Figs. 7 and 8), which does not require Ca²⁺ (Masuda and de Meis, 1973). Contrary to Ca²⁺ binding, however, enzyme phosphorylation with Pₐ previous to addition of TG, affords no apparent protection from inactivation by TG. Furthermore, depending on the experimental conditions, TG inhibition of the Pₐ reaction is not complete, but is only increased by TG through interference with the inhibition by Pi, those on Ca²⁺ occlusion by the CrATPase. In accordance with the experiments on enzyme phosphorylation with Pi previous to addition of TG, the level of phosphorylation by Pi and the level of phosphorylation by Ca²⁺ are actually increased by TG through interference with activation by Pi, those on Ca²⁺ occlusion by the CrATPase. In conclusion, our experiments indicate that TG reacts with external Ca²⁺ and, therefore, is unable to utilize ATP (Figs. 6). This indicates that EGTA reacts with external Ca²⁺ and TG reacts with the enzyme only following spontaneous dissociation of occluded Ca²⁺, consequently preventing further association events. Therefore, TG can react with the enzyme only after dissociation of occluded Ca²⁺.

In conclusion, our experiments indicate that TG reacts with a conformational state acquired by the ATPase in the absence of Ca²⁺, and produces total inhibition of high affinity binding of external Ca²⁺. Owing to interference with activation by Ca²⁺, the TG·ATPase complex is unable to utilize ATP in the forward direction of the cycle.

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