Unravelling the Interaction of Thapsigargin with the Conformational States of Ca\(^{2+}\)-ATPase from Skeletal Sarcoplasmic Reticulum\(^*\)

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Preincubation of thapsigargin with sarcoplasmic reticulum vesicles in the presence of high Ca\(^{2+}\) or the addition of high Ca\(^{2+}\) to microsomal vesicles preincubated with thapsigargin in the absence of Ca\(^{2+}\) allowed full enzyme phosphorylation by ATP. However, the enzyme activity was not protected by high Ca\(^{2+}\) even when the samples were subjected to gel filtration before ATP addition. Our data indicate that: (i) the enzyme in the Ca\(^{2+}\)-bound conformation can be stabilized in the presence of thapsigargin; (ii) the conformational transition from the Ca\(^{2+}\)-free to the Ca\(^{2+}\)-bound state can be elicited by Ca\(^{2+}\) when thapsigargin is present; (iii) thapsigargin binding occurs whether or not the enzyme is in the presence of Ca\(^{2+}\), and so a ternary complex enzyme-Ca\(^{2+}\)-thapsigargin may be formed; (iv) thapsigargin can be dissociated from the enzyme with a slow kinetics after dilution under drastic conditions; (v) the kinetics of Ca\(^{2+}\) binding is clearly slowed down by thapsigargin; and (vi) thapsigargin does not affect the hydrolysis rate of phosphorylating substrates when measured in the absence of Ca\(^{2+}\), indicating that thapsigargin specifically inhibits the Ca\(^{2+}\)-dependent activity.

Thapsigargin (TG) is a naturally occurring sesquiterpene lactone of the guianolide type (1, 2) that can be found in the root of several species of the genus Thapsia (3). It was initially described as a tumor promoting agent with ability to induce rapid Ca\(^{2+}\) release from intracellular stores (4). The effect was later recognized to be indirect since TG inhibits the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum membrane (5-7). It was also established that equimolar TG with respect to the enzyme concentration is sufficient to produce complete inhibition of the ATP-dependent Ca\(^{2+}\) transport (6). Nowadays, TG is a key molecule for investigating the selective mobilization of Ca\(^{2+}\) from the intracellular reticulum.

A combined approach including kinetics and molecular biology techniques as well as structural data have provided important clues on the inhibitory effect of TG. For instance, it has been shown that TG inhibits Ca\(^{2+}\) binding in the absence of ATP and enzyme phosphorylation by inorganic phosphate in the absence of Ca\(^{2+}\) (8). Likewise, the inhibition has been related to the Ca\(^{2+}\)-free conformation of the enzyme (6, 8, 9). Some transient protection by Ca\(^{2+}\) when EP was formed from ATP and the accumulation of a dead end complex as a consequence of the enzyme turnover has also been observed (10). The interaction of TG and Ca\(^{2+}\) with the enzyme was described as involving mutual exclusion when a low free Ca\(^{2+}\) medium was used and the interaction of TG with the enzyme was reported as being irreversible when the experiments were performed at 25 °C (10).

As regards the location of the TG-binding domain, the use of a fluorescent TG derivative (11) or photolabeling with a radioactive azido derivative of TG (12) indicated that the binding occurs within or near the transmembrane region of the enzyme. More precise information was obtained by chimeric construct and site-directed mutagenesis after overexpression of the Ca\(^{2+}\)-ATPase protein in COS-1 cells. These studies revealed that the TG-binding domain does not reside within the large cytoplasmic loop (13). Location at the membrane interface and interaction with the S3 stalk segment between Asp\(^{274}\) and Leu\(^{280}\) have been suggested (14). The S3 segment has also been related with the binding domain of the high affinity inhibitor cyclopiazonic acid (15). In this sense, the region around Phe\(^{256}\), where the structural elements M3, M4, and L67 gather, seems to be critical for Ca\(^{2+}\) binding (16). It is envisioned that the presence of TG might destabilize certain hydrogen bonds and, as a consequence, decrease Ca\(^{2+}\) binding affinity (16).

We have now performed a systematic and in-depth study which will help to elucidate the interaction of TG with the enzyme and shed light on the inhibition mechanism of this highly specific inhibitor of the SR Ca\(^{2+}\)-ATPase.

EXPERIMENTAL PROCEDURES

Materials—TG was obtained from Molecular Probes Europe. A23187 from Streptomyces chartreusensis was obtained from Calbiochem. Stock solutions of TG or A23187 were prepared in dimethyl sulfoxide (ACS reagent). The Ca\(^{2+}\) standard solution (Titrisol) was a product of Merck. \([\gamma\text{-}^{32}\text{P}]\text{ATP was purchased from PerkinElmer Life Sciences. The liquid scintillation mixture (S4023) and other reagents of analytical grade, including deoxycholate (D4297), were from Sigma. HAWP filter units with a pore diameter of 0.45 μm were from Millipore. Samples were manually filtered under vacuum in a Hoefer filtration box (Amersham Pharmacia Biotech).}\)

Sample Preparation—Fast-twitch leg muscle obtained from adult female New Zealand rabbit was the starting material. A microsomal fraction enriched in longitudinal tubules of the SR membrane was prepared by differential centrifugation according to Eletr and Inesi (17). Purified Ca\(^{2+}\)-ATPase was obtained by method 2 of Meissner et al. (18). Fig. 1 shows protein staining of the preparations after SDS-electrophoresis (19). Samples were frozen in liquid nitrogen and kept at −80 °C until use. The protein concentration was estimated by the
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Fig. 1. Electrophoretic profile of samples used in this study. Samples were subjected to SDS electrophoresis (19) and then stained with Coomassie Blue. The major protein components in the SR membrane are: Ca\textsuperscript{2+}-ATPase (CA), calsequestrin (CS), and the 53-kDa glycoprotein (GP) (lane 2). CS, GP, and other contaminating proteins were absent after Mg\textsuperscript{2+} ATPase purification (lane 3). Molecular mass markers from top to bottom are: myosin, \( \beta \)-galactosidase, phosphorylase b, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme (lane 1).

colorimetric procedure of Lowery et al. (20) using bovine serum albumin as standard. One mg of SR protein contains ~4 nmol of active enzyme, as deduced from the maximal phosphorylation level, so that a vesicular protein concentration of 0.2 mg/ml is equivalent to 0.8 \( \mu \text{M} \) Ca\textsuperscript{2+}-ATPase.

Free Ca\textsuperscript{2+} in Media—The ionized free Ca\textsuperscript{2+} concentration was fixed by adding given volumes of CaCl\textsubscript{2} and/or EGTA stock solutions. Theoretical values were calculated by a computer program as previously described (21). The procedure takes into consideration the absolute stability constant for the Ca\textsuperscript{2+}-EGTA complex (22), the pH values for the EGTA protonation (23), pH, and the presence of relevant ligands in the medium.

Preincubation with TG before Phosphorylation—SR vesicles in the absence of free Ca\textsuperscript{2+} was named the \( E_0 \) medium and consisted of 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\textsubscript{2}, 0.1 mM EGTA, 0.2 mg/ml SR vesicles, and 15 \( \mu \text{M} \) A23187. The \( E_0 \text{Ca}\textsubscript{2} \) (10 \( \mu \text{M} \)) medium was prepared by adding 0.105 mM CaCl\textsubscript{2} to the Ca\textsuperscript{2+}-free medium, whereas the \( E_0\text{Ca}_2 \) (3 mM) medium contained 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl\textsubscript{2}, 0.1 mM EGTA, 0.2 mg/ml SR vesicles, 15 \( \mu \text{M} \) A23187, and 3.1 mM CaCl\textsubscript{2}. Aliquots of 0.5 mL containing \( E_0 \) or \( E_0\text{Ca}_2 \) samples were preincubated at 25 \( ^\circ \text{C} \) for 5 min with TG. The phosphorylation of \( E_0\text{Ca}_2 \) after preincubation with TG was preceded by a brief incubation at 0 \( ^\circ \text{C} \) for 1 min. Phosphorylation of \( E_0 \) plus TG required the preliminary addition of 0.105 mM CaCl\textsubscript{2} (10 \( \mu \text{M} \) free Ca\textsuperscript{2+}) and the subsequent incubation at 0 \( ^\circ \text{C} \) for 1 min. In all cases, samples were phosphorylated at 0 \( ^\circ \text{C} \) with 10 \( \mu \text{M} \) [\( \gamma \text{32P}\text{]}\text{ATP as described below.}

Accumulated EP after TG Preincubation—The phosphorylation reaction was initiated at 0 \( ^\circ \text{C} \) by adding 10 \( \mu \text{M} \) [\( \gamma \text{32P}\text{]}\text{ATP (50,000 cpm/}

mm) were preincubated at 25 \( ^\circ \text{C} \) for 5 min with 0.8 \( \mu \text{M} \) TG. Then, 3.1 mM CaCl\textsubscript{2} (3 mM free Ca\textsuperscript{2+}) was added and the incubation was prolonged at 0, 25, or 37 \( ^\circ \text{C} \) for different time intervals. Samples were cooled for 1 min in an ice bath before phosphorylation at 0 \( ^\circ \text{C} \) for 1 s, as described before.

Incubation with TG or Ca\textsuperscript{2+} before Gel Filtration—Initial samples were 1-ml aliquots of the following media: \( E_0 \), \( E_0\text{TG} \) (i.e. \( E_0 \) preincubated at 25 \( ^\circ \text{C} \) for 5 min with 0.8 \( \mu \text{M} \) TG) or \( E_0\text{Ca}_3 \) (3 mM). Samples of \( E_2 \) medium were used as a control (without any addition) or were incubated at 25 \( ^\circ \text{C} \) for 5 min with 0.8 \( \mu \text{M} \) (equimolar) TG. \( E_0\text{TG} \) samples were incubated at 37 \( ^\circ \text{C} \) for 1 h with 3.1 mM Ca\textsuperscript{2+} (3 mM free Ca\textsuperscript{2+}), whereas \( E_0\text{Ca}_3 \) (3 mM) was incubated at 25 \( ^\circ \text{C} \) for 5 min with 0.8 \( \mu \text{M} \) TG. Samples of \( E_0 \) or \( E_0\text{Ca}_2 \) incubated with TG (0.55 mM) were centrifuged at room temperature for 1 min through small chromatography columns (38 \( \times \) 10 mm) filled with Sephadex G-50 resin and pre-equilibrated in a Ca\textsuperscript{2+}-free medium (20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\textsubscript{2}, and 0.1 mM EGTA). Likewise, samples of \( E_0\text{TG} \) incubated with Ca\textsuperscript{2+} or \( E_0\text{Ca}_3 \) (3 mM) incubated with TG (0.55 mM) were centrifuged through Sephadex columns pre-equilibrated in a Ca\textsuperscript{2+}-free medium (20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl\textsubscript{2}, 0.1 mM EGTA, and 3.1 mM CaCl\textsubscript{2}). In all cases, the Sephadex columns were centrifuged at low speed for 1 min before use as previously described (24). The filtrate from each column was diluted with 5 ml of medium to reach a final free Ca\textsuperscript{2+} of 50 \( \mu \text{M} \). The dilution medium for \( E_2 \) samples or \( E_0\text{Ca}_3 \) was incubated with TG was 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\textsubscript{2}, 0.1 mM EGTA, 1.1 mM phosphoenolpyruvate, 6 units/ml pyruvate kinase, and 1.5 \( \mu \text{M} \) A23187. The dilution medium for \( E_0\text{TG} \) incubated with 3 mM Ca\textsuperscript{2+} or \( E_0\text{Ca}_3 \) (3 mM) incubated with TG was 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 0.22 mM Cl\textsubscript{2}, 1.1 mM phosphoenolpyruvate, 6 units/ml pyruvate kinase, and 1.5 \( \mu \text{M} \) A23187. The enzyme activity was measured at 37 \( ^\circ \text{C} \) in 1-ml aliquots of diluted samples. The ATP concentration was 50 \( \mu \text{M} \) and the procedure described by Lin and Morales was followed (25). Ca\textsuperscript{2+}-ATPase Activity in the Presence of TG without Dilution—The initial incubation medium (1.5 ml) contained 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\textsubscript{2}, 0.1 mM EGTA, and 1 \( \mu \text{M} \) A23187. When indicated, 30 nM TG was also included. The incubation medium was maintained at 37 \( ^\circ \text{C} \) for 5 min. The rate of Ca\textsuperscript{2+}-ATPase activity was measured at 37 \( ^\circ \text{C} \) by the method described by Lanzetta et al. (26) after addition of 0.15 mM CaCl\textsubscript{2}, 1 mM phosphoenolpyruvate, 6 units/ml pyruvate kinase, and 50 \( \mu \text{M} \) ATP. Aliquots of 0.2 ml were withdrawn at different times and mixed with 0.8 ml of color reagent. The color development was stopped after 1 min by adding 0.1 ml of 34\% sodium citrate and the absorbance at 660 nm was read 30 min later. A molar ratio was varied in the range of 0.5 to 4. The hydrolytic reaction was stopped (usually after 1 s) by the addition of 50 \( \mu \text{M} \) A23187. The dilution medium for \( E_2\text{TG} \) incubated with 3 mM Ca\textsuperscript{2+} was 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\textsubscript{2}, 0.1 mM EGTA, and 3.1 mM CaCl\textsubscript{2}.
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*p*-nitrophenyl phosphate. The release of inorganic phosphate from the nucleotides was evaluated by the procedure of Lin and Morales (25). The hydrolysis of *p*-nitrophenyl phosphate was followed by measuring the accumulation of *p*-nitrophenol (27).

**Data Presentation**—Each plotted value corresponds to the average of at least three independent assays performed in duplicate. The standard deviation (plus or minus) for each assay is given.

**RESULTS**

The inhibitory effect of TG can be conveniently assessed by studying the capacity of the enzyme to be phosphorylated by ATP in the presence of Ca$^{2+}$. Initially, SR vesicles in a 10 μM free Ca$^{2+}$ medium were preincubated at 25 °C for 5 min with equimolar TG. Samples were subsequently transferred to an ice bath for 1 min and then 1–20-s phosphorylation was achieved by the sequential addition of 10 μM [γ-32P]ATP and acid quenching solution. An accumulation of approximately 0.5 nmol of P/mg of protein from the initial time point was observed (closed circles in Fig. 2A). As expected, the enzyme was fully phosphorylated when TG was not added during preincubation (open circles). A different pattern was observed when equimolar TG was added to SR vesicles in a 3 mM free Ca$^{2+}$ medium. In this case, the EP accumulation showed an initial rise followed by a progressive decrease as the phosphorylation time was prolonged (closed squares in Fig. 2B). The time course of EP formed under the same conditions but without TG added during preincubation is shown as a control (open squares).

The transient protection by millimolar Ca$^{2+}$ was also analyzed by changing the free Ca$^{2+}$ added during preincubation and evaluating EP after brief phosphorylation (1 s at 0 °C with 10 μM [γ-32P]ATP). When equimolar TG was added to vesicles in a Ca$^{2+}$-containing medium and the incubation lasted 5 min at 25 °C, EP increased as the Ca$^{2+}$ concentration was raised (closed squares in Fig. 3A). Maximal phosphorylation was obtained at 100 μM free Ca$^{2+}$, although a tendency to decrease was observed at higher Ca$^{2+}$ concentrations. Control experiments performed in the absence of TG exhibited a decrease of EP from 3.2 to 2.1 when the pCa was raised from 5.5 to 2.5 (open squares). The TG effect is also shown as a percentage of EP versus pCa (closed squares in Fig. 3B). Closed triangles in Fig. 3B correspond to data obtained when the preincubation with equimolar TG was maintained for 1 h at 37 °C. It is apparent that half-maximal protection by Ca$^{2+}$ decreased as the preincubation time was prolonged and the temperature was raised.

The effect of TG was also studied when the enzyme was initially in the E2 conformation. To this end, SR vesicles in a nominally Ca$^{2+}$-free medium were supplemented at 25 °C with a given TG concentration. Then, Ca$^{2+}$ was added to reach a final 10 μM free concentration and the preincubation was maintained at 25 °C for 5 min. The TG effect was evaluated after 1 min incubation in an ice bath and 1 s phosphorylation at 0 °C by 10 μM [γ-32P]ATP. Preincubation of E2TG with 10 μM free Ca$^{2+}$ produced a TG-dependent inhibition of the accumulated EP (Fig. 4). EP was completely inhibited by 0.8 μM TG when the SR protein was 0.2 mg/ml, i.e. when the Ca$^{2+}$-ATPase was 0.8 μM. This means that complete inhibition was observed when the TG/enzyme molar ratio was ≥1.

The protective role of millimolar Ca$^{2+}$ when added to E2TG was studied as follows: SR vesicles in a Ca$^{2+}$-free medium were preincubated at 25 °C for 5 min with equimolar TG and then Ca$^{2+}$ was added to reach a final 3 mM free concentration. The incubation was prolonged at 0, 25, or 37 °C and the samples taken at different times after adding Ca$^{2+}$ were used to evaluate EP (1 s phosphorylation at 0 °C). Thus, the EP level increased in the minute time scale and was clearly dependent on temperature (Fig. 5). The accumulation rate was very low when the incubation temperature in the presence of Ca$^{2+}$ was 0 °C (open triangles). The rate was higher at 25 °C and EP reached values close to maximum when the Ca$^{2+}$ incubation was maintained for 60 min (open squares). The recovery was even faster when the temperature was raised to 37 °C (open circles). Samples with no TG added during preincubation were also phosphorylated at 37 °C and in a 3 mM free Ca$^{2+}$ medium and are shown as a reference (closed circles).

The interaction of TG and Ca$^{2+}$ with the enzyme was more deeply explored by using different preincubation protocols before the samples were subjected to gel filtration and the Ca$^{2+}$-ATPase activity was measured (Fig. 6). In all cases, the enzyme activity was measured at 37 °C in the same reaction medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl$_2$, 0.1 mM EGTA, 0.105 mM CaCl$_2$, 0.2 mg/ml SR vesicles, and 15 μM A23187 or the E2Ca$_2$(3 mM) medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl$_2$, 0.1 mM EGTA, 3.1 mM CaCl$_2$, 0.2 mg/ml SR vesicles, and 15 μM A23187 were preincubated at 25 °C for 5 min with 0.8 μM TG. Samples were further incubated in an ice bath for 1 min before the addition of 10 μM [γ-32P]ATP. The phosphorylation reaction was stopped at the times plotted in the figure. A, EP formed from E2Ca$_2$(10 μM) in the absence (C) or presence (D) of 0.8 μM TG. B, EP formed from E2Ca$_2$(3 mM) in the absence (□) or presence (■) of 0.8 μM TG.

![Fig. 2. Time course of accumulated EP after preincubation of E$_2$Ca$_2$ with equimolar TG at low or high Ca$^{2+}$](http://www.jbc.org/)

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**Fig. 2. Time course of accumulated EP after preincubation of E$_2$Ca$_2$ with equimolar TG at low or high Ca$^{2+}$.** The E$_2$Ca$_2$(10 μM) medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl$_2$, 0.1 mM EGTA, 0.105 mM CaCl$_2$, 0.2 mg/ml SR vesicles, and 15 μM A23187 or the E$_2$Ca$_2$(3 mM) medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl$_2$, 0.1 mM EGTA, 3.1 mM CaCl$_2$, 0.2 mg/ml SR vesicles, and 15 μM A23187 were preincubated at 25 °C for 5 min with 0.8 μM TG. Samples were further incubated in an ice bath for 1 min before the addition of 10 μM [γ-32P]ATP. The phosphorylation reaction was stopped at the times plotted in the figure. A, EP formed from E$_2$Ca$_2$(10 μM) in the absence (C) or presence (D) of 0.8 μM TG. B, EP formed from E$_2$Ca$_2$(3 mM) in the absence (□) or presence (■) of 0.8 μM TG.
from the column, the sample was diluted in the reaction medium containing 50 \( \mu \)M free Ca\(^{2+} \) and the enzyme activity was measured. After this treatment the rate of ATP hydrolysis was 3 mol/min/mg of protein. However, the enzyme activity was only 0.14 when \( E_2 \) was preincubated at 25 °C for 5 min with equimolar TG. The Sephadex column pre-equilibrated in the absence of Ca\(^{2+} \) was unable to eliminate the inhibition even though the enzyme activity was measured in the 50 \( \mu \)M free Ca\(^{2+} \) medium described before. In another set of experiments, \( E_2 \) was formed by preincubating (25 °C for 5 min) aliquots of 0.5 ml containing \( E_2 \) medium in the presence of 20 mM MgCl\(_2\) with 0.8 \( \mu \)M TG. After the addition of 3.1 mM CaCl\(_2\) (zero time) the incubation was prolonged at 0 (\( E_2 \)), 25(\( C \)), or 37 °C (\( O \)). Samples were cooled at fixed time intervals in an ice bath for 1 min and then phosphorylated at 0 °C for 1 s with 10 \( \mu \)M \( [\gamma-\text{32P}] \)ATP. The reaction was stopped after 1 s by the addition of 5 ml of quenching solution. Samples were processed to correlate radioactive EP with TG concentration in preincubation.

Under our assay conditions, that included 0.01 mg/ml SR

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FIG. 3. Dependence on Ca\(^{2+}\) of the EP formed after preincubation of \( E_1 \) with equimolar TG. Aliquots of \( E_1 \) in the presence of a given Ca\(^{2+}\) concentration (0.5 ml) were preincubated with 0.8 \( \mu \)M TG. Samples were cooled in an ice bath for 1 min and then phosphorylated for 1 s with 10 \( \mu \)M \( [\gamma-\text{32P}] \)ATP. A, EP formed in the absence of TG (\( \square \)), or after preincubation at 25 °C for 5 min with 0.8 \( \mu \)M TG (\( \blacksquare \)). B, EP, in relative units, after preincubation at 25 °C for 5 min (\( \square \)) or 37 °C for 1 h (\( \triangle \)) in the presence of TG. Details on preincubation media and EP measurement are provided under “Experimental Procedures.”

FIG. 4. EP formed after preincubation of \( E_2 \) with \( \mu \)M Ca\(^{2+}\). Aliquots of 0.5 ml containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 0.1 mM EGTA, 0.2 mg/ml SR vesicles, and 15 \( \mu \)M A23187 were equilibrated at 25 °C with a given TG concentration. Then, 0.105 mM CaCl\(_2\) (10 \( \mu \)M free Ca\(^{2+}\)) was added and the incubation was maintained at 25 °C for 5 min. The mixture was transferred to an ice bath for 1 min and the phosphorylation was started at 0 °C by adding 10 \( \mu \)M \( [\gamma-\text{32P}] \)ATP. The reaction was stopped after 1 s by the addition of 5 ml of quenching solution. Samples were processed to correlate radioactive EP with TG concentration in preincubation.

FIG. 5. Time dependence of EP formed from ATP after preincubation of \( E_2 \) with mM Ca\(^{2+}\). \( E_2 \) was formed by preincubating (25 °C for 5 min) aliquots of 0.5 ml containing \( E_2 \) medium in the presence of 20 mM MgCl\(_2\) with 0.8 \( \mu \)M TG. After the addition of 3.1 mM CaCl\(_2\) (zero time) the incubation was prolonged at 0 (\( E_2 \)), 25(\( C \)), or 37 °C (\( O \)). Samples were cooled at fixed time intervals in an ice bath for 1 min and then phosphorylated at 0 °C for 1 s with 10 \( \mu \)M \( [\gamma-\text{32P}] \)ATP. EP formed after addition at 37 °C of 3 mM free Ca\(^{2+}\) to \( E_2 \) in the absence of TG (\( O \)).
protein and 50 μM ATP, half-maximal inhibition was observed at 19 nM TG (data not shown). Using the conditions of this experiment we studied the putative reversibility of TG. Leaky SR vesicles (0.01 mg/ml) in the absence of Ca\(^{2+}\) were first equilibrated with 30 nM TG and then diluted and incubated for a certain time span. Aliquots taken after dilution were used to evaluate Ca\(^{2+}\)-ATPase activity. All the manipulations were carried out at 37 °C and the dilution medium contained 50 μM free Ca\(^{2+}\). Control experiments were performed with TG in preincubation. Data in Fig. 7A correspond to samples diluted to different degrees and incubated at 37 °C for 2 h. It is clear that enzyme activity recovery was dependent on the dilution factor (open bars). Data are expressed on a relative scale and the 100% value at each dilution corresponds to the enzyme activity of diluted samples measured at 37 °C using 50 μM ATP as a substrate and an ATP-regenerating system.

The addition of [γ-\(^{32}\)P]ATP to E\(_{1}\)Ca\(_{2}\) leads to the formation of radioactive EP by transfer of the ATP γ-phosphate to the enzyme. Therefore, the E\(_{1}\)Ca\(_{2}\) conformation, as opposed to E\(_{2}\), can be functionally distinguished by a brief phosphorylation at 0 °C. Our initial EP measurements confirmed previously reported inhibitory effects of TG, namely the sensitivity of the E\(_{2}\) reaction cycle proceeded (6, 8–10) and some protection by Ca\(^{2+}\) (10).

This study reveals that the ATP phosphorylating capacity was dependent on Ca\(^{2+}\) concentration when equimolar TG was added to E\(_{2}\)Ca\(_{2}\) (Figs. 2 and 3). These experiments required 1–2 s phosphorylation at 0 °C since longer phosphorylation times led to EP dephosphorylation and the accumulation of E\(_{2}\)TG as the reaction cycle proceeded (6, 10) (see also Fig. 2B). The inhibition degree was also dependent on incubation time and temperature (Fig. 3B). Higher Ca\(^{2+}\) concentrations were needed to observe a certain EP level when the incubation was prolonged from 5 min to 1 h and the temperature was raised from 25 to 37 °C. These data are consistent with intrinsic fluorescence......
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**SCHEME I. Interaction of TG with the Ca$^{2+}$-dependent conformations of the enzyme.** TG can interact and bind to the $E_1$Ca$_2$ or $E_2$ conformations. The stabilization of $E_1$Ca$_2$TG or $E_2$TG is dependent on Ca$^{2+}$ concentration. The transitions induced in our study were: transition a (Figs. 2B and 3), transition a plus c (Figs. 2A and 3), transition b (Fig. 5), and transition d plus e (Fig. 7).

The recovery of enzyme activity after dilution (Fig. 7) demonstrates that TG can be dissociated from the enzyme and, therefore, that it is reversibly bound. The dissociation was observed after a 100-fold dilution when the protein concentration was lowered to 0.1 µg/ml and TG was 0.3 nM. The dissociation kinetics was very slow and required a long incubation at 37°C (Fig. 7B). The high temperature was critical since no dissociation was observed when the incubation was performed at 25°C even though similar conditions after dilution were used (10). Experiments in Fig. 7 correspond to the transition $E_2$TG → $E_2$ + TG → $E_1$Ca$_2$ in Scheme I.

The hydrolysis of phosphorylating substrates in the absence of Ca$^{2+}$ was unaffected by the presence of TG even when used above the equimolar level and was sustained by the purified enzyme (Fig. 8). This indicates that TG does not affect the catalytic properties of $E_2$. The normal operation of the reaction cycle in a Ca$^{2+}$-containing medium involves the participation of $E_1$Ca$_2$, $E_3$, and phosphorylated species. When the reaction cycle takes place in the presence of Ca$^{2+}$ and TG is also present, the enzyme is stacked as $E_1$TG. Under these conditions the $E_1$TG complex in the presence of Ca$^{2+}$ exhibits the same hydrolytic activity as the enzyme in the absence of Ca$^{2+}$ and TG. It should be recalled that the residual activity measured in the presence of Ca$^{2+}$ coincided with that measured in the absence of Ca$^{2+}$ when samples were pretreated with TG (Fig. 6). This confirms that the inhibitory effect of TG is on the Ca$^{2+}$-dependent but not on the Ca$^{2+}$-independent activity. Inhibition by TG has also been shown to be dependent on the $E_1$Ca$_2$/Ca$^{2+}$ ratio when p-nitrophenyl phosphate was the substrate (27).

Full phosphorylation by ATP in the presence of equimolar TG and millimolar Ca$^{2+}$ was observed using leaky SR (Figs. 2 and 3) and was also reproduced in native vesicles (data not shown) indicating that Ca$^{2+}$ binding to the external (high affinity) sites is sufficient to exert the protective effect. Saturating the internal (low affinity) Ca$^{2+}$ sites has also been reported to decrease the inhibitory effect of TG on enzyme phosphorylation from inorganic phosphate (10). These data suggest that, under equilibrium conditions, any action directed at decreasing the accumulation of $E_2$TG has a protective role. In contrast, we have checked that the Ca$^{2+}$-dependent activity is inhibited by TG when millimolar Ca$^{2+}$ is present in the external and/or internal medium. This confirms that Ca$^{2+}$ binding to the cytoplasmic or luminal sites does not provide protection when the enzyme is cycling.

**FIG. 8. Hydrolytic activity in a Ca$^{2+}$-free medium of $E_2$ samples (purified enzyme) preincubated with TG.** Purified Ca$^{2+}$-ATPase (0.4 mg/ml) in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl$_2$, and 1 mM EGTA were preincubated at 25°C for 5 min with a given TG concentration. The release of phosphate at 25°C was measured after the addition of 1 mM ATP (■), 1 mM UTP (▲), or 10 mM p-nitrophenyl phosphate (▲).
The interaction of TG and Ca\(^{2+}\) with the enzyme in the absence of ATP is summarized in Scheme I. The binding of TG is not dependent on the conformational state of the enzyme and therefore \(E_1\text{Ca}_2\) and \(E_2\) are targets for TG. The stabilization of \(E_1\text{Ca}_2\text{TG}\) or \(E_2\text{TG}\) under equilibrium conditions is dependent on the Ca\(^{2+}\) concentration present. This study demonstrates that the equilibria among the Ca\(^{2+}\)-dependent conformations of the enzyme in the presence of TG can be shifted by ligand addition and/or sample dilution.

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