A Conformational Mechanism for Formation of a Dead-end Complex by the Sarcoplasmic Reticulum ATPase with Thapsigargin

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(Received for publication, August 8, 1991)

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Thapsigargin (TG), a plant sesquiterpene lactone extract, interacts tightly with the sarcoplasmic reticulum (SR) Ca\(^{2+}\) transport ATPase yielding a 1:1 stoichiometric complex. In addition to inhibiting steady state enzyme activity, TG can be shown to inhibit two individual partial reactions of the ATPase cycle (i.e. Ca\(^{2+}\) binding in the absence of ATP and enzyme phosphorylation by Pi, in the absence of Ca\(^{2+}\)) even when these reactions are studied separately without interdependence. As the two partial reactions occur at domains relatively distant from each other in the protein structure, it is apparent that the TG induced perturbation involves the entire enzyme. The rate of TG interaction with the ATPase, as estimated by the onset of functional inhibition and by the development of an intrinsic fluorescence signal, is relatively low in the presence of Ca\(^{2+}\). The interaction is much faster when Ca\(^{2+}\) is removed from the medium by the addition of EGTA or is dissociated from the enzyme by utilization of ATP. When the TG interaction with the ATPase is studied in the presence of Ca\(^{2+}\) as a function of temperature (15–35 °C) and pH (6.0–8.0), two distinct kinetic components are observed: a fast component which is prevalent at high temperature and low pH, and a slow component which is prevalent at low temperature and high pH. This pattern suggests that the enzyme resides in two states, whose relatively slow equilibration is temperature- and pH-dependent. As only one state is reactive to TG, the enzyme population residing in this state reacts immediately with TG. On the other hand, the enzyme population residing in the alternate state must undergo slow conversion to the reactive state before being affected by TG. It can also be demonstrated that in the presence of Ca\(^{2+}\) TG shifts the ATPase from a refractory state to a state which is able to form bidimensional crystalline arrays stabilized by cationic detergent. It is concluded that TG reacts specifically with the ATPase conformation which is prevalent in the absence of Ca\(^{2+}\), thereby forming a catalytically inactive dead-end complex.

Exp. Biol. Med. 205, 1286 (1991)

Thapsigargin (TG), a tumor-promoting sesquiterpene lactone, has been shown to raise the intracellular concentration of Ca\(^{2+}\) in several cell lines (Jackson et al., 1988; Scharff et al., 1988). This effect was attributed to TG interference with the endoplasmic reticulum regulatory function (Thastrup et al., 1990; Thastrup, 1990). It was subsequently shown (Sagara and Inesi, 1991) that the Ca\(^{2+}\) transport activity of sarcoplasmic reticulum (SR) vesicles purified from skeletal muscle is inhibited by extremely low concentrations of TG, with a stoichiometric ratio of 1 mol of inhibitor per mol of ATPase. Inhibition is produced equally well on various isoforms of intracellular Ca\(^{2+}\) transport ATPases, obtained by transfection of mammalian cells with specific cDNAs (Campbell et al., 1991). TG is much less effective on the plasma membrane Ca\(^{2+}\) transport ATPase and not effective at all on Ca\(^{2+}\) channels that are dependent on Ca\(^{2+}\) plus ATP (Sagara and Inesi, 1991), phosphoinositide metabolism, or protein kinase C (Jackson et al., 1988).

Owing to its high affinity and stoichiometric effectiveness, TG is of particular interest with respect to its molecular interaction with the SR Ca\(^{2+}\)-ATPase, and its interference with the partial reactions of the catalytic and transport cycle. To this end we consider, at first approximation, the following partial reactions:

\[
\begin{align*}
E + 2\text{Ca}^{2+}(\text{out}) & \leftrightarrow E \cdot \text{Ca}_2 \\
E \cdot \text{Ca}_2 + \text{ATP} & \leftrightarrow E \cdot \text{P} \cdot \text{Ca}_2 + \text{ADP} \\
E \cdot \text{P} \cdot \text{Ca}_2 & \leftrightarrow E \cdot \text{P} + 2\text{Ca}^{2+} \\
E \cdot \text{P} & \leftrightarrow E + \text{P}_i
\end{align*}
\]

We find that, at the same concentration which inhibits steady state activity, TG inhibits two individual reaction components of the catalytic cycle, i.e. Ca\(^{2+}\) binding in the absence of ATP (reaction 1, above), and enzyme phosphorylation with Pi in the absence of Ca\(^{2+}\) (reaction 4, above). We studied these two reactions separately under equilibrium conditions and in the absence of ATP, using radioactive tracer and spectrophotometric methods. The fluorescence signals of intrinsic tryptophan and fluorescent ATPase labels were used to monitor thapsigargin binding and its possible conformational effects on the enzyme.

EXPERIMENTAL PROCEDURES

Thapsigargin (TG) was purchased from LC Service Corp., Woburn, MA. Fluorescein 5'-isothiocyanate (FITC) and IAEDANS were purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma. Vesicular fragments of longitudinal sarcoplasmic reticulum (SR) were obtained from rabbit skeletal muscle according to Eletr and Inesi (1972), and the protein concentration was measured by the method of Lowry et al. (1951) standardized with bovine serum albumin.

Derivatization of SR ATPase with FITC and IAEDANS was carried out as described by Bigelow and Inesi (1991). It has been previously established that, under those conditions, FITC reacts with
Lys-515 (Mitchison et al., 1989) and IAEKDNS with Cys-670 and -674 (Bishop et al., 1988) of the ATPase, without loss of activity.

Steady state ATP hydrolysis by SR ATPase was tested in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mM EGTA, and 10 μg of SR protein/ml. The Ca²⁺-independent partial reaction mixture of the reaction mixture to render the SR vesicles leaky, thereby avoiding "back inhibition" by a rise of Ca²⁺ in the lumen of the vesicles. Following addition of ATP (2.5 mM), serial samples were taken for determination of P₁ according to Lin and Morales (1977).

The phosphorylated enzyme intermediate produced by utilization of ATP was obtained by mixing 1.0 mg of SR protein in 2.0 ml of a medium containing 50 mM MES-TRIS, pH 6.2, 10 mM MgCl₂, 20% (v/v) Me₂SO, 2 mM EGTA, and 0.5 mM [³²P]P. Following a 15-min incubation at 25 °C, the reaction was quenched with perchloric acid and the denatured protein was treated as described above.

Calcium binding (in the absence of ATP) to the SR ATPase was measured by equilibration in a chromatography column (62.5 × 0.55 cm) filled with Sephadex G-75 resin (40 to 120 mesh). The equilibrium medium contained 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂, and 10 μM [³⁵Ca]CaCl₂. An aliquot of SR vesicles (1.0 mg of protein) suspended in 0.2 ml of a medium containing 20 mM MOPS, pH 7.0, and 10% sucrose, was added on top of the gel and eluted in approximately 60 min at room temperature. Fractional samples were collected for determination of protein and radioactive calcium. The excess calcium over the baseline, eluted with the protein peak, was considered to be bound calcium (Inesi et al., 1989). Alternatively, 80 μg of SR protein were placed on a Millipore (0.45 μm) filter fitted on a BioLogic rapid filtration apparatus. The loaded filters were perfused for 600 ms with a medium identical with that described above. The filter was then collected and processed for determination of radioactive calcium. The radioactivity remaining with filters perfused with the same medium in the absence of SR was used as the background.

Fluorescence measurements were carried out on a Photon Technology International spectrophotometer (Alpha Scan), using a 150-watt xenon lamp. The reaction mixture, containing 0.05–0.10 mg of SR protein/ml and other additions specified in the legends to the figures, was placed in a temperature-controlled, 2-ml cuvette, and steady-state fluorescence intensity was monitored continuously. Excitation and emission wavelengths are specified in figure legends.

Samples to be examined by electron microscopy contained 0.4–0.5 mg of SR protein/ml, in a medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂ and other additions as required by the experimental schedule. In some cases, the samples were incubated for 5 min with 5 mM decavanadate prepared by titrating a 100 mM stock solution of Na₂V₁₀O₁₄ to pH 2–3. For negative staining, samples were applied to glow-discharged carbon-coated grids, which were then washed with four changes of buffer and stained with 1% uranyl acetate. The specimens were examined with a Zeiss 10 CA electron microscope at 80 kV accelerating voltage.

RESULTS

Inhibition of ATPase Partial Reactions by TG — In a previous study (Sagara and Inesi, 1991), we found that inhibition of steady state Ca²⁺ transport and ATPase activity is produced by thapsigargin with a 1:1 stoichiometry with SR ATPase. This stoichiometric match was independent of the ATPase concentration, indicating a very strong affinity of the inhibitor for the enzyme. We considered that, in principle, inhibition of steady state activity could be produced by interference with a single partial reaction of the catalytic and transport cycle. Unexpectedly, when we measured individually two distinct reactions of the enzyme cycle, i.e. Ca²⁺ binding in the absence of ATP (reaction 1 of the scheme in the introduction) and formation of phosphorylated intermediate with P₁ in the absence of Ca²⁺ (reaction 4 of the scheme in the introduction), we found that interaction of one molecule of thapsigargin with one molecule of ATPase affects both partial reactions as well as the steady state activity (Fig. 1). This suggests that the inhibition by TG is not limited to a single site, but rather encompasses the enzyme as a whole.

The measurements reported in Fig. 1 were obtained under equilibrium conditions. We then determined the time required for functional inactivation of the ATPase by TG. To this end, SR vesicles were incubated with TG in the presence of 10 μM Ca²⁺ at 25 °C for various times and were then tested for the ability of the ATPase to bind Ca²⁺ in the absence of ATP. We found that, under these conditions, inhibition of Ca²⁺ binding was produced rather slowly, over a period of minutes (Fig. 2, A). On the other hand, if the functional competence of the ATPase was tested at 35 °C by measuring its ability to form phosphorylated enzyme intermediate upon addition of ATP, functional inactivation occurred much faster (not shown). In fact, resolution of the time course of inactivation (when tested by enzyme phosphorylation with ATP) could be resolved better if the incubation was carried out in ice (Fig. 2, O, , and C). It is of interest that a much faster inactivation was observed if the phosphorylation reaction with ATP was carried out for 10, rather than 1 s (Fig. 2, , and O, respectively). Finally, we found when the incubation of the SR vesicles with TG was carried out in the absence of Ca²⁺ (EGTA added), inactivation occurred very rapidly even at the lower temperature (Fig. 2, C). These experiments indicate that ATPase inactivation occurs much faster when the incubation with TG is carried out in the absence of Ca²⁺ and at a relatively high temperature. It is apparent then that TG reacts with the ATPase when Ca²⁺ is dissociated from the enzyme due to the presence of EGTA in the medium or as a consequence of ATP utilization (see also Sagara and Inesi, 1991). The question of the Ca²⁺ and temperature dependence of the TG interaction with the SR ATPase was pursued more conveniently and in greater detail by continuous measurements.

![Fig. 1. Inhibition of Ca²⁺ binding (), enzyme phosphorylation with P₁ ( ), and steady state ATPase activity () by TG. Ca²⁺ binding was measured by equilibration of SR vesicles with a medium containing 20 mM MOPS, pH 7, 80 mM KCl, 5 mM MgCl₂, and 10 μM [³⁵Ca]CaCl₂, temperature 25 °C. Enzyme phosphorylation with P₁ was measured following equilibration of SR vesicles with a medium containing 50 mM MES-TRIS, pH 6.2, 10 mM MgCl₂, 20% (v/v) Me₂SO, and 2 mM EGTA. Temperature, 25 °C. Steady state ATPase activity was measured in a reaction mixture containing 20 mM MOPS, pH 7, 80 mM KCl, 5 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mM EGTA, 10 μg of SR protein/ml, and 2 μM concentration of the ionophore A23187. Temperature, 25 °C. Previous to starting these reactions, SR vesicles were incubated for 5 min at 25 °C with the amount of TG specified in the figure.](image-url)
of intrinsic fluorescence, as explained below.

Detection of TG Effects by Fluorescence Measurements—Considering that Ca++ binding or dissociation yields fluorescence signals originating from the ATPase tryptophan residues (Dupont, 1976), we thought that it should be possible to monitor conveniently the effect of TG by measurements of intrinsic fluorescence. It is shown in Fig. 3A (upper trace) that if Ca++ is added to SR ATPase in the presence of EGTA, to yield a free Ca++ concentration of approximately 1 μM, the intrinsic fluorescence is increased. If TG is then added, the fluorescence change is reversed due to dissociation of bound Ca++ from the ATPase. On the other hand, if TG is added to SR ATPase in the presence of EGTA and no added Ca++, a reduction of intrinsic fluorescence is also observed (lower trace). This indicates that binding of TG generates its own fluorescence reduction, in addition to the reduction related to Ca++ displacement.

In addition to the fluorescence rise produced by Ca++ binding, it is known that phosphorylation of SR ATPase with Pi, in the absence of Ca++ (Masuda and de Meis, 1973) is also followed by a change of intrinsic fluorescence (Lacapere et al., 1981). It is shown in Fig. 3B (upper trace) that such an effect of Pi is reversed by TG, consistent with inhibition of the Pi reaction and decay of the phosphoenzyme level. If TG is added first, the fluorescence effect of Pi is not observed (Fig. 3B, lower trace).

It was reported by Pick and Bassilian (1981) that FITC label of Lys-515 in the SR ATPase (Mitchinson et al., 1982) results in a corresponding reduction of its fluorescence intensity following Ca++ binding, in parallel with the rise of intrinsic fluorescence. We found that the effect of Ca++ binding on the fluorescence of the FITC label is prevented (Fig. 4A, upper trace) or reversed by TG (Fig. 4A, lower trace), consistent with the changes observed in intrinsic fluorescence (Fig. 3).
On the other hand, in the presence of EGTA and in the absence of added Ca\(^{2+}\), neither enzyme phosphorylation with P\(_t\), nor TG by itself, affects the fluorescence of the FITC label (Fig. 4B). In parallel experiments, we found that the fluorescence intensity of an iodoacetamide-directed label (IAE-DANS) of Cys-670 and -674 (Bishop et al., 1988) is not affected either by Ca\(^{2+}\) or TG (not shown).

**Effect of Ca\(^{2+}\) on the Onset of the TG Effect**—During the course of our experimentation, we noted that the onset of TG inhibition was delayed by the presence of Ca\(^{2+}\) in the medium (Fig. 2). This delay was more systematically demonstrated by monitoring the ATPase intrinsic fluorescence, as shown in Fig. 5A. These experiments were started in a reaction mixture containing 9 \(\mu\)M endogenous calcium. Addition of 20 \(\mu\)M EGTA produced in all cases a reduction of intrinsic fluorescence due to displacement of Ca\(^{2+}\) from the specific ATPase sites. Soon after EGTA, increasing concentrations of Ca\(^{2+}\) were added in parallel runs, yielding no fluorescence signal in the uppermost trace (due to chelation by the excess EGTA present), and then stepwise fluorescence rises as the aliquot of added Ca\(^{2+}\) was increased up to the saturation level of the ATPase sites. Following saturation, the fluorescence rise remained constant even when the concentration of added Ca\(^{2+}\) was increased further, demonstrating that this signal is only related to occupancy of the specific sites.

It is also shown in Fig. 5A that the Ca\(^{2+}\)-induced fluorescence signal is reversed by TG at a progressively lower rate as the Ca\(^{2+}\) concentration in the medium is higher. On the other hand, if thapsigargin is added first, (Fig. 5B), subsequent additions of Ca\(^{2+}\) up to 0.5 mM do not reverse the effect of TG. This indicates that the TG-ATPase complex is not destabilized by Ca\(^{2+}\), even though the rate of its formation is influenced by the presence of Ca\(^{2+}\).

**pH and Temperature Dependence of the TG Effect**—A most interesting set of observations was made when displacement of Ca\(^{2+}\) from the specific ATPase sites by EGTA or TG was monitored by measurements of intrinsic fluorescence, as a function of pH and temperature. It is shown in Fig. 6 that displacement of Ca\(^{2+}\) by EGTA is fast, being manifested by a sharp reduction of intrinsic fluorescence, independent of pH from 6.0 to 8.0, and temperature from 25 to 35 °C. On the contrary (Fig. 7), the time course of the TG effect changes from a fast response at pH 6 and 35 °C, to an extremely slow change at pH 8.0 and 15 °C. Most importantly, it is apparent that under intermediate conditions the TG response is biphasic, including a fast and a slow component. The ratio between fast and slow components is highest at low pH and high temperature and lowest at high pH and low temperature. The rate of the slow component is also reduced by raising the pH and reducing the temperature.

It should be noted that previous measurements by radioactive tracer demonstrated that, at the same pH, saturation of the high affinity sites by Ca\(^{2+}\) is not changed by temperature (Watanabe et al., 1981). Yet, at the same pH, the ratio of fast and slow components of fluorescence change is highly temperature-dependent. This indicates that the enzyme containing bound Ca\(^{2+}\) resides in two states of different sensitivity to

**FIG. 5.** Effects of Ca\(^{2+}\) on the intrinsic fluorescence change produced by TG (A) and of TG on the change produced by Ca\(^{2+}\) (B). A. 0.1 mg of SR protein/ml was incubated in 2 ml of a medium containing 20 mM MOPS, pH 7, 80 mM KCl, 5 mM MgCl\(_2\). 20 \(\mu\)M EGTA, various concentrations of CaCl\(_2\), and 0.8 \(\mu\)M TG were then added at 30-s intervals. B. 0.1 mg of SR protein/ml was incubated in 2.0 ml of a medium identical with that described in A. 20 \(\mu\)M EGTA, 0.8 \(\mu\)M TG, and 0.2 or 0.5 mM CaCl\(_2\) were then added at 30-s intervals. Temperature, 25 °C, \(A_{\text{ex}}\) = 290 nm; \(A_{\text{em}}\) = 330 nm (vertical axis, photon counting units).

**FIG. 6.** Temperature and pH dependence of the EGTA effect on intrinsic fluorescence. A medium containing 20 mM Tris/Bis-Tris (adjusted to pH 6, 7, or 8), 80 mM KCl, 5 mM MgCl\(_2\), and 9 \(\mu\)M Ca\(^{2+}\) was pre-equilibrated to achieve 15, 25, or 35 °C. 0.2 mg of SR protein was then added to 2.0 ml of the pre-equilibrated medium, and 0.1 mM EGTA was added after 60 s. \(A_{\text{ex}}\) = 290 nm; \(A_{\text{em}}\) = 330 nm (vertical axis, photon counting units).
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TG. Equilibration of the two states is relatively slow and temperature-dependent.

Measurements of TG Binding to the SR ATPase—The intrinsic fluorescence signal observed following addition of TG in the presence of EGTA is related to a direct effect of the inhibitor on the ATPase and provides a convenient method to monitor directly TG binding to the enzyme. A titration of the enzyme with TG is shown in Fig. 8, yielding a progressive reduction of fluorescence intensity as TG is added to match the enzyme stoichiometry (1 mg of SR protein contains 5 nmol of enzyme, as determined independently by titration of phosphorylated intermediate). No further fluorescence reduction is observed upon subsequent additions of TG, demonstrating directly the saturation character of TG binding (Fig. 9). As binding does not differ significantly from the amount of TG added, it is apparent that the binding affinity is very high, although a precise figure cannot be obtained. Previous titrations of the inhibitory effect provided a more sensitive (albeit indirect) indication of binding affinity, suggesting a value higher than $10^9$ M$^{-1}$ (Sagara and Inesi, 1991).

Bidimensional Crystalline Arrays of the ATPase: Effects of Ca$^{2+}$ and TG—Under appropriate conditions, the SR ATPase acquires a bidimensional crystalline order consisting of dimer ribbon arrays closely packed within the plane of the membrane (Buhle et al., 1983; Castellani et al., 1985; Dux and Martonosi, 1983). SR vesicles obtained from rabbit muscle require decavanadate for stabilization of the ordered arrays (Coan et al., 1986), while SR vesicles prepared from scallop muscle do not (Castellani et al., 1985). However, a common requirement for formation of ordered arrays is that Ca$^{2+}$ be removed from the ATPase, which is usually accomplished by the addition of EGTA to the medium (Fig. 10A). Arrays do not form if decavanadate is added in the presence of Ca$^{2+}$ (Fig. 10B). This indicates that the Ca$^{2+}$-free enzyme acquires a special conformation which permits bidimensional crystalline ordering. We now find that, in the presence of Ca$^{2+}$, addition of TG renders the enzyme able to acquire such a bidimensional crystalline order (Fig. 10C). In the absence of decavanadate, neither the presence of EGTA nor the presence of TG (Fig. 10D) promotes ordering of the ATPase.

DISCUSSION

Previous studies on SR ATPase derivatization with relatively small organic molecules have resulted in specific inhibition of one of the partial reactions of the catalytic cycle. For instance, derivatization of the transmembrane domain with a fluorescent carbodiimide derivative (NCD4) inhibits specifically Ca$^{2+}$ binding, but not the phosphorylation reac-
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All samples contained 0.5 mg of SR protein/ml, 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂, and 0.2 mM EGTA. In addition, specific samples contained: A, 5 mM decavanadate; B, 0.25 mM CaCl₂ and 5 mM decavanadate; C, 0.25 mM CaCl₂, 0.1 μM TG, and 5 mM decavanadate; D, 0.25 mM CaCl₂ and 0.1 μM TG. All samples were prepared for electron microscopy as described under “Experimental Procedures.”

Inhibition is due to sequestration of the enzyme in a dead-end complex with TG, with consequent inability to form

Additional evidence indicating that the interaction of TG with SR ATPase is selective with respect to the conformational state of the ATPase, is provided by the Ca²⁺ interference with the onset of thapsigargin inhibition. This is demonstrated by the experiment shown in Fig. 2, in which the ability of the enzyme to bind Ca²⁺ and form the phosphorylated enzyme intermediate is tested with ATP and Ca²⁺, at various time intervals following addition of TG to SR ATPase preincubated with or without Ca²⁺. It is clear that the onset of TG inhibition is immediate when the SR ATPase is preincubated without Ca²⁺, while the inhibition occurs with considerable delay when Ca²⁺ is present. In other experiments where TG and ATP were added simultaneously to SR preincubated with Ca²⁺, the delay for the onset of inhibition was relatively short (Sagara and Inesi, 1991), due to rapid interaction of TG with the ATPase state resulting from Ca²⁺ dissociation at the end of each cycle of ATP utilization. It is apparent that TG interacts with the ATPase deprived of Ca²⁺ either by EGTA or as a consequence of ATP utilization. Inhibition is then due to sequestration of the enzyme in a dead-end complex with TG, with consequent inability to form

**Fig. 10.** Effect of TG on the ability of the ATPase to form bidimensional ordered arrays. All samples contained 0.5 mg of SR protein/ml, 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂, and 0.2 mM EGTA. In addition, specific samples contained: A, 5 mM decavanadate; B, 0.25 mM CaCl₂ and 5 mM decavanadate; C, 0.25 mM CaCl₂, 0.1 μM TG, and 5 mM decavanadate; D, 0.25 mM CaCl₂ and 0.1 μM TG. All samples were prepared for electron microscopy as described under “Experimental Procedures.”

**Fig. 11.** Diagram representing structural features and points of reference in the SR ATPase. The predicted secondary structure (MacLennan et al., 1985; Green et al., 1986) includes 10 transmembrane helices, the segments protruding from the membrane, and the site of tryptic cleavage yielding the large tryptic fragments. The residues (Asp-351) undergoing phosphorylation and derivatization with FITC (Lys-515) or IAEDANS (Cys-670 and -674) are also shown in the extramembranous region. All the tryptophans are indicated by the letter W, showing their prevalent location within or near the transmembrane domain of the enzyme. The Ca²⁺ binding domain is located within the transmembrane region (Clarke et al., 1989; Sumbilla et al., 1991).
the phosphorylated intermediate and to enter further catalytic cycles. All these findings can be illustrated:

\[
\begin{align*}
\text{EGTA} & \rightarrow \text{EGTA} - \text{Ca}^{2+} \\
\text{2 Ca}^{2+} & \rightarrow \text{E-Ca}_{2} \\
\text{ATP} & \rightarrow \text{E} \rightarrow \text{E-P-Ca}_{2} \\
\text{2 Ca}^{2+} & \rightarrow \text{E-TG} \\
\text{E} & \rightarrow \text{F} \\
\text{E-P} & \rightarrow \text{2 Ca}^{2+}
\end{align*}
\]

where TG reacts with E when Ca\(^{2+}\) is not present in the medium (i.e. presence of EGTA), or when Ca\(^{2+}\) is removed from the enzyme as a consequence of ATP utilization. The E-TG complex is then very stable and does not allow the enzyme to enter further cycles.

The intrinsic fluorescence of the ATPase tryptophan turned out to be a very convenient signal to monitor the binding of TG. In fact, the reduction of fluorescent intensity observed following addition of TG to SR vesicles in the presence of Ca\(^{2+}\) is partly due to Ca\(^{2+}\) dissociation from its high affinity sites (Fig. 7A). On the other hand, the further reduction of fluorescent intensity observed when TG is added following chelation of endogenous Ca\(^{2+}\) with EGTA, is actually due to binding of the inhibitor to the ATPase. This demonstrates that TG binds to the ATPase directly and that saturation is reached (Fig. 9) when the stoichiometry of the ATPase is equimolar to the added TG.

In the presence of Ca\(^{2+}\), the effect of TG is rather slow, and its onset is delayed by a relatively high pH and low temperature. If the time course of the fluorescence change is studied as a function of pH and temperature, a biphasic pattern is noted under conditions permitting saturation of the high affinity sites by Ca\(^{2+}\) (Fig. 7). The fast component becomes more prominent as the pH is lowered and the temperature raised, while the slow component becomes more prominent as the pH is raised and the temperature lowered. This pattern suggests that the ATPase resides in two states, one of which is more receptive, and the other less receptive to thapsigargin. The equilibrium between the two states is shifted in favor of the more receptive state as the pH is raised and the temperature lowered. The equilibrium between the two states may be written as

\[
\begin{align*}
*E & \leftrightarrow E \\
E + 2\text{Ca}^{2+} & \leftrightarrow E\cdot\text{Ca}_{2}
\end{align*}
\]

as proposed by de Meis and Vianna (1979), or as

\[
\begin{align*}
E + 2\text{Ca}^{2+} & \leftrightarrow E\cdot\text{Ca}_{2} \\
E\cdot\text{Ca}_{2} & \leftrightarrow E\cdot\text{Ca}_{2}
\end{align*}
\]

as proposed by Inesi et al. (1990). In these sequences, the \(E \leftrightarrow E\) and the \(E\cdot\text{Ca}_{2} \leftrightarrow E\cdot\text{Ca}_{2}\) represent slow isomorphic transitions, yielding two distinct conformational states of the enzyme. In the context of our present experiments, we tend to favor the latter scheme, as detection of the two states requires the presence of Ca\(^{2+}\), and the ratio between the two states can be changed by varying the temperature while the Ca\(^{2+}\) sites remain saturated (Fig. 7B). At any rate, it is clear that TG reacts with the enzyme state devoid of Ca\(^{2+}\), which is known to be reactive to P, (Masuda and de Meis, 1973; de Meis and Masuda, 1974) and to be favored by low pH and high temperature. The temperature dependence of the equilibrium between two states of the SR ATPase exhibiting different features with respect to function (Inesi et al., 1976) and physical state (Birmachu and Thomas, 1990) was considered previously in detail.

The fluorescence changes undergone by the ATPase tryptophan residues suggest that thapsigargin produces a conformational change in the ATPase region where Ca\(^{2+}\) is bound. This does not mean necessarily that TG binds in the same region, nor that the conformational change is limited to that region. We note, however, that all but one of the ATPase tryptophan residues are thought to reside within or near the transmembrane region (Fig. 11). Measurements of fluorescence intensity decay by frequency modulation methods have shown that the fluorescence enhancement produced by Ca\(^{2+}\) is due to an increase of the distribution and average lifetimes of the ATPase tryptophan residues (Gryczynski et al., 1989), likely related to stabilization of transmembrane helices by Ca\(^{2+}\) binding.

The fluorescence signal of the FITC label of Lys-515, which resides in the extramembranous region of the ATPase (Fig. 11), is also affected by Ca\(^{2+}\) displacement by TG (Fig. 4), while the signal of the IAEDANS labels of Cys-670 and -674, is not affected. Most importantly, the Ca\(^{2+}\) -free enzyme state which is stabilized by TG is sufficiently different from the Ca\(^{2+}\)-bound state, as to make an all or none difference with respect to formation of bidimensional crystalline order by decanaradate (Fig. 10). It is of interest that in analogy to the long range conformational coupling of Ca\(^{2+}\) binding and phosphorylation domains during the functional catalytic cycle, a long range linkage of these two domains is also manifested in the mechanism of TG inhibition.

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