Self-Association Accompanies Inhibition of Ca-ATPase by Thapsigargin

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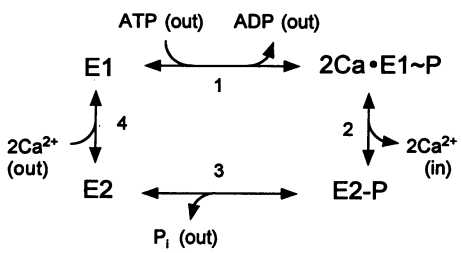
ABSTRACT Recent studies have demonstrated a relationship between the activity of the Ca-ATPase of sarcoplasmic reticulum and its state of self-association. In the present study, the effects of thapsigargin (TG), a toxin that specifically inhibits the Ca-ATPase of rabbit skeletal muscle sarcoplasmic reticulum membrane, were studied by detecting the time-resolved phosphorescence anisotropy (TPA) decay of the Ca-ATPase that had been labeled with the phosphorescent probe erythrosin-isothiocyanate (ErITC). Anisotropy decays were fit to a function that consisted of three exponential decays plus a constant background, as well as to a function describing explicitly the uniaxial rotation of proteins in a membrane. In the absence of TG, the anisotropy was best-fit by a model representing the rotation of three populations, corresponding to different-sized oligomeric species in the membrane. The addition of stoichiometric amounts of TG to the Ca-ATPase promptly decreased the overall apparent rate of decay, indicating decreased rotational mobility. A detailed analysis showed that the principal change was not in the rates of rotation but rather in the population distribution of the Ca-ATPase molecules among the different-sized oligomers. TG decreased the proportion of small oligomers and increased the proportion of large ones. Preincubation of the ErITC-SR in 1 mM Ca^{2+}, which stabilizes the E1 conformation relative to E2, was found to protect partially against the changes in the TPA associated with the presence of the inhibitor. These results are consistent with the hypothesis that TG inhibits the Ca-ATPase by stabilizing it in an E2-like conformation, which promotes the formation of larger aggregates of the enzyme. When combined with the effects of other inhibitors on the Ca-ATPase, these results support a general model for the coupling of enzyme conformation and self-association in this system.

INTRODUCTION

The Ca-ATPase of skeletal sarcoplasmic reticulum (SR) is a 110 kDa intrinsic membrane protein that translocates Ca^{2+} ions from the cytoplasmic side of the membrane to the luminal side, initiating the relaxation of contracted muscle. A widely accepted mechanism for this reaction is illustrated in Scheme 1. In the E1 state, the enzyme binds 2 calcium ions



Scheme 1

and one molecule of ATP from the cytoplasmic side ("out") with high affinity. (1) ATP is hydrolyzed to ADP, and the energy of the phosphate bond is transferred to the enzyme with the formation of the phosphoenzyme E1P. (2) A conformational change isomerizes the phosphoenzyme from the high energy E1P state to the lower energy E2P state, the calcium affinity decreases, and the Ca^{2+} ions are released on the luminal side ("in"). (3) The enzyme is dephosphorylated to E2. (4) Another conformational change returns the enzyme to the E1 state, which is facilitated by Ca^{2+} binding. A fundamental question about the Ca-ATPase concerns its mechanism of regulation. It is of interest to know what changes in protein structure or oligomeric state might modulate Ca-ATPase activity in response to a change in the enzyme's environment.

A number of studies have investigated the effect of external agents on the microsecond rotational motion of the Ca-ATPase, as measured either by saturation transfer electron paramagnetic resonance (ST-EPR) or time-resolved phosphorescence anisotropy (TPA). The general anesthetics diethyl ether (Bigelow and Thomas, 1987; Birmachu and Thomas, 1990) and halothane (Karon and Thomas, 1993) were shown to increase the rotational mobility of the labeled Ca-ATPase while activating the enzyme. The bee-venom peptide melittin decreased the rotational mobility (Mahaney and Thomas, 1991; Voss et al., 1991) while inhibiting the enzymatic activity. The time resolution of TPA, combined with EPR measurements of lipid fluidity, showed that these changes in rotational motion were primarily due to changes in the oligomeric state of the Ca-ATPase, and not to changes in lipid fluidity. Throughout these and other studies (reviewed by Thomas and Mahaney, 1993; Thomas and Karon, 1994), those agents that increased the protein rotational mobility (implying a shift to smaller oligomers of the Ca-ATPase) also caused the activity to increase, and those agents that decreased the rotational mobility (corresponding to the formation of larger oligomers) also decreased activity. Agents that increase the self-association of the Ca-ATPase

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also appear to stabilize the E2 conformational state (Karon et al., 1994; Voss et al., 1993, 1995)

To probe further the possible coupling between the conformational transition and protein self-association in this system, it is desirable to have a specific means of shifting the E1-E2 conformational equilibrium. This requirement is met by thapsigargin (TG), a sesquiterpene lactone, which has been used in recent years to increase cytosolic calcium levels in a number of different types of cells including human platelets (Tao and Haynes, 1992) and vascular smooth muscle cells (Xuan et al., 1992). Earlier studies showed that TG is a specific inhibitor of the SERCA Ca-ATPase family (Sagara and Inesi, 1991; Lytton et al., 1991), with complete inhibition occurring in the SR with sub-micromolar affinity at a stoichiometry of 1 mole of TG per mole of Ca-ATPase monomer (Kijima et al., 1991; Sagara and Inesi, 1991). Binding of TG results in release of one calcium ion while ATP remains bound, suggesting that TG inhibits the enzyme by binding at or near one of the high affinity calcium binding sites. TG binding is accompanied by changes in the intrinsic tryptophan fluorescence of the ATPase, as well as in the fluorescence of a covalently bound NBD label. Because the changes in the fluorescence intensity of NBD-Cl-labeled SR induced by TG are similar to those induced by vanadate, and because vanadate is known to bind to and stabilize the E2 conformation, TG is thought to "lock" the enzyme into an E2-like conformation (Wictome et al., 1992, Sagara et al., 1992b). The high specificity of TG for the Ca-ATPase makes it a valuable agent for studying the coupling between the E1-E2 equilibrium and other physical parameters of the system.

Optical spectroscopy has proven to be a useful technique for the study of the binding of TG to the Ca-ATPase and the resulting conformational changes (Sagara et al., 1992a; Wictome et al., 1992), but the changes in the fluorescence intensity seen in these studies have not led to a specific structural model for the physical and functional consequences of TG binding. The technique of TPA, however, is capable of monitoring protein oligomeric state, which has been shown to correlate with Ca-ATPase activity (Thomas and Karon, 1994). Therefore, in the present study we have used TPA spectroscopy to test whether changes in the Ca-ATPase oligomeric state play a role in the inhibition induced by TG, a coupling that has been recognized in a number of other enzymes (Traut, 1994). Our results provide new insight into the mechanism of action of this specific inhibitor and into a potential physical mechanism of regulation of the Ca-ATPase in SR membranes.

MATERIALS AND METHODS

Reagents and solutions

Erythrosin 5′-isothiocyanate (ErITC) was obtained from Molecular Probes (Eugene, OR). Thapsigargin was obtained from LC Services (Woburn, MA). Both ErITC and TG were dissolved in DMF and stored in liquid nitrogen before use. Catalase, glucose oxidase, β-D glucose, pyruvate kinase, lactate dehydrogenase, phosphoenol pyruvate, ATP, and NADH were purchased from Sigma Chemical Co. (St. Louis, MO). A23187 was obtained from Calbiochem (San Diego, CA). All biochemical and spectroscopic measurements were carried out at 25°C in a standard buffer containing 20 mM MOPS, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1 mM EGTA at pH 7.0.

Preparations and assays

Sarcoplasmic reticulum (SR) vesicles were prepared from New Zealand White rabbit fast-twitch skeletal white muscle according to the method of Fernandez et al. (1980), and purified on a discontinuous sucrose gradient (Birmachu et al., 1989) to remove the heavy SR vesicles that consisted of junctional SR and contained calcium release proteins. The light SR was harvested and suspended in a solution of 0.3 M sucrose and 30 mM MOPS at pH 7.0, and stored in liquid nitrogen until use. Protein concentration was determined by the Biuret method (Gornall et al., 1949) using bovine serum albumin as a standard. Densitometry of SDS gels showed that the Ca-ATPase constitutes approximately 80 ± 5% of the protein by weight (Birmachu and Thomas, 1990). Using 110 kDa as the molecular weight of the Ca-ATPase, our preparations consisted of 7.0 ± 0.4 nmol Ca-ATPase/mg protein.

Ca-ATPase activity was assayed by an enzyme-linked, continuous ATP assay as described by Karon and Thomas (1993). Between 5 and 50 μg/ml of Ca-ATPase in SR vesicles was added to a buffer containing 2 mM phosphoenolpyruvate, 0.24 mM NADH, 120 IU of pyruvate kinase, and 120 IU of lactate dehydrogenase. The assay mixture also contained 1 μg/ml of the ionophore A23187 to prevent a buildup of calcium inside the vesicles that might inhibit the Ca-ATPase activity. ATP (2 mM) was added to start the assay, and the absorbance of NADH was followed at 340 nm to determine the amount of ATP hydrolyzed. ATP hydrolysis of the SR vesicles was fully coupled to calcium transport.

TPA data acquisition

For phosphorescence experiments, the Ca-ATPase was specifically labeled at lysine 515 with ErITC as described previously (Birmachu and Thomas, 1990). The reaction of 1.0 ± 0.2 mol of dye per mole of Ca-ATPase results in greater than 90% inhibition of activity, confirming the complete and specific reaction of ErITC with Lys-515 on the Ca-ATPase. After labeling, samples were diluted to a concentration of 0.25 mg/ml in the standard buffer. Oxygen was removed from the sample by the addition of 100 μg/ml glucose oxidase, 15 μg/ml catalase, and 5 mg/ml β-D glucose (Eads et al., 1984). Deoxygenation was allowed to proceed for 10 min before data collection. Aliquots (1–5 μl) of TG dissolved in DMF were added directly to the sample, which was then briefly vortexed. Any oxygen that may have entered the sample upon addition of the inhibitor was found not to change measurably the probe luminescence lifetime. After adding inhibitor, samples were allowed to incubate for 15 min, and the TPA was then measured.

The anisotropy is defined as

$$r(t) = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}}, \quad (1)$$

where I_{vv} and I_{vh} are the vertical and horizontal components of the emission after excitation with a vertically polarized pulse. TPA decays were recorded using an instrument described previously (Ludescher and Thomas, 1988), by signal-averaging the time-dependent phosphorescence decays with a single detector and a polarizer that alternates between vertical ($I_{vv}(t)$) and horizontal ($I_{vh}(t)$) orientation every 2000 flashes. G is an instrumental correction factor, determined by measuring the anisotropy of a solution of free dye under experimental conditions and adjusting G to give an anisotropy value of zero, the theoretical value for a freely tumbling chromophore.

Data analysis

TPA decays were analyzed by fitting them to theoretical expressions, using the nonlinear least-squares algorithm of Marquardt (Bevington, 1969). The quality of the fit was gauged by comparing χ^2 values and by evaluating the residuals. Previous reports from this laboratory (Birmachu and Thomas, 1990; Cornea and Thomas, 1994) have shown that the TPA of ErITC-SR

is dominated by the uniaxial rotational diffusion of the labeled Ca-ATPase about an axis normal to the bilayer, and that it is a good approximation to fit the data to a sum of n exponentially decaying terms plus a constant background term,

$$\frac{r(t)}{r(0)} = \sum_{i=1}^{n} A_i e^{-t/\phi_i} + A_\infty, \tag{2}$$

where ϕ_i, the rotational correlation time of the ith component, is inversely proportional to the rotational diffusion coefficient D_i. A_∞ is the normalized residual anisotropy $r_\infty/r(0)$, given by

$$A_\infty = A_{\infty_0} + f_I \times (1 - A_{\infty_0}), \tag{3}$$

where f_I is the fraction of proteins that are immobile on the observed time scale and A_{∞_0} is the residual anisotropy that results from the anisotropic rotation of the mobile fraction about the normal to the membrane. The fraction of probes (proteins) with a correlation time ϕ_i is thus given by $f_i = A_i/(1 - A_{\infty_0})$. The value of A_{∞_0} depends on the orientation of the probe transition dipoles with respect to the membrane normal (see Fig. 1), and it is given by:

$$A_{\infty_0} = \frac{P_2(\cos\theta_a)P_2(\cos\theta_e)}{P_2(\cos\delta)}, \tag{4}$$

(Lipari and Szabo, 1980; Cornea and Thomas, 1994) where $P_2(x) = (3x^2 - 1)/2$. Here θ_e is the angle between the emission dipole and the normal to the membrane, θ_a is the angle between the absorption dipole and the normal to the membrane, and δ is the angle between the absorption and emission transition dipoles (see Fig. 1).

In the present study, we have also fit the data to the exact analytical function that Eq. 2 approximates, which incorporates all of the terms that describe the anisotropy of uniaxially rotating chromophores:

$$r(t) = \sum_{i=1}^{n} f_i r_i(t) + f_I r(0), \tag{5}$$

where f_i is the mole fraction associated with the ith rotating species. The expression for the individual anisotropy of this ith uniaxially rotating species is given by (Szabo, 1984)

$$r_i(t) = \kappa\,(a_1 e^{-D_i t} + a_2 e^{-4D_i t} + a_3), \tag{6}$$

with

$$a_1 = \tfrac{6}{5}\sin\theta_a\cos\theta_a\cos\phi_{ae}\cos\theta_e\sin\theta_e, \tag{7a}$$

$$a_2 = \tfrac{3}{10}\sin^2\theta_a\sin^2\theta_e\cos 2\phi_{ae}, \tag{7b}$$

$$a_3 = \tfrac{2}{5}P_2(\cos\theta_e)P_2(\cos\theta_a). \tag{7c}$$

Here D_i is the rotational diffusion coefficient about the normal to the membrane and ϕ_{ae} is the azimuthal angle between the absorption and emission transition dipoles of the probe. The initial anisotropy is given by

$$r(0) = \tfrac{2}{5}\kappa P_2(\cos\delta), \tag{8}$$

where $\cos\delta = \cos\theta_a\cos\theta_e + \sin\theta_a\sin\theta_e\cos\phi_{ae}$. κ is the order parameter for fast (sub-microsecond) rotation of the probe with respect to the protein and is given in general form by

$$\kappa = \langle P_2\cos\Delta\theta\rangle^2, \tag{9}$$

where $\Delta\theta$ describes the amplitude of the motion and the brackets indicate an ensemble average. This motion may result from the site of probe attachment (Lys-515) undergoing segmental motion or from partial freedom of motion of the probe itself around the site of attachment. It can be modeled as "wobble-in-a-cone" type motion, in which the probe is free to move within a cone of half-angle θ_c centered about an axis determined by the probe's local environment. In this case, κ is given by (Kinosita et al., 1977)

$$\kappa = [\tfrac{1}{2}\cos\theta_c(1 + \cos\theta_c)]^2. \tag{10}$$

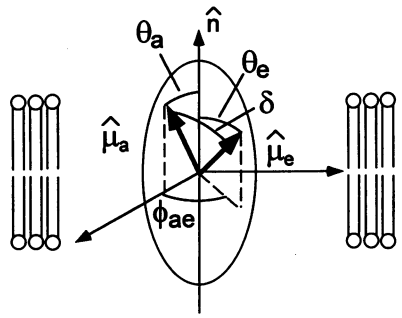


FIGURE 1 An illustration of the angles appearing in Eqs. 4 and 7a–c. μ_a and μ_e are vectors pointing in the direction of the absorption and emission transition dipole, respectively, of the probe molecule. θ_a and θ_e are the polar angles of the absorption and emission dipoles with respect to the membrane normal. ϕ_{ae} is the azimuthal angle of μ_e, with the x-y plane defined such that the azimuthal angle of μ_a is = 0. δ is the angle between the dipoles.

The more complete model (described by Eqs. 5–7) differs from our previous analysis (described by Eq. 2) only in (1) the inclusion of the biexponential decay for each species (Eq. 6), (2) the explicit inclusion of the fast wobble parameter κ, and (3) the explicit inclusion of the angular dependence of the amplitudes of the exponentially decaying terms. This type of analysis has been applied to the Ca-ATPase by Restall et al. (1984), who modeled the TPA decay as a single species rotating uniaxially while the probe undergoes wobble-in-a-cone diffusion within the protein itself. The use of a single species was found to be inadequate to describe the curves in the present report, however, and our best results (evaluated from residuals and χ^2 values) were obtained by using a function that included three rotating species. Each species was assumed to have the same values of ϕ_{ae}, θ_a, and θ_e, and to differ from the other species only in its diffusion coefficient, presumably because of differences in aggregation state.

The rotational diffusion coefficient of these rotating species is predicted by the Saffman-Delbrück equation:

$$D = kT/(4\pi a^2 h\eta), \tag{11}$$

where a is the effective radius of the protein in the bilayer, h is the height of the protein in contact with the membrane, k is Boltzmann's constant, η is the effective membrane viscosity, and T is the temperature. Thus, the rotational diffusion coefficient is proportional to the lipid fluidity (T/η, Squier et al., 1988b) and inversely proportional to the intramembrane cross sectional area (πa^2) of the protein. An increase in lipid fluidity or a decrease in the effective radius of the rotating species will cause an increase in the protein's rotational diffusion coefficient. This has been confirmed by previous studies on the Ca-ATPase using ST-EPR (Squier et al., 1988a) and phosphorescence anisotropy (Birmachu and Thomas, 1990). The latter study showed that correlation times obtained by fitting TPA decays can be used to determine the sizes of Ca-ATPase oligomers, and normalized amplitudes can be used to determine the mole fractions of oligomers present in SR.

RESULTS

The TPA decay of ErITC Ca-ATPase is depicted in the bottom curve of Fig. 2. Also depicted is the TPA 15 min after the addition of increasing amounts of TG, up to a concentration of 3.5 μM, which corresponds to a stoichiometry of 2:1 TG:ATPase. The inset shows an expanded view of the region between 75 and 100 μs. Addition of TG causes a small but consistent and easily detected change in the TPA curve up to the level of 7 nmol TG/mg ATPase (1:1 stoichiometry), above which no further change in the curve is seen. Many previous reports have shown that a stoichiometric amount of TG is enough to inhibit completely the enzyme within 15 min

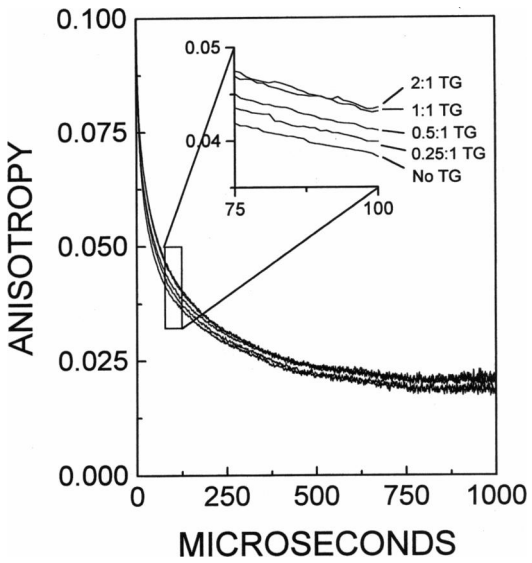


FIGURE 2 The TPA curves of ErITC-labeled Ca-ATPase at 25°C in the presence of increasing amounts of TG. The inset shows that TG decreases the apparent rate of decay of the anisotropy until a ratio of 1:1 TG:ATPase, above which no further change occurs.

(Sagara et al., 1992a, Kijima et al., 1991; Wictome et al., 1992). Therefore, inhibition is associated with an increase in the anisotropy, and this has previously been linked to enzyme aggregation (reviewed by Thomas and Karon, 1994).

To determine whether TG causes these TPA changes by decreasing Ca-ATPase rotational mobility caused by a decrease in SR bilayer fluidity (T/η, Eq. 11), we used EPR spectroscopy to measure lipid hydrocarbon chain mobility in the presence of these inhibitors (Squier et al., 1988b). The SR bilayer was spin-labeled with a stearic acid derivative labeled at the C-5 position, which is sensitive to fluidity near the headgroup of the bilayer (Mahaney and Thomas, 1991). TG (up to 2 mol TG per mole Ca-ATPase) had less than one percent effect on the order parameter measured from the EPR spectral splittings (data not shown), indicating that observed TPA changes are not due to changes in SR bilayer fluidity.

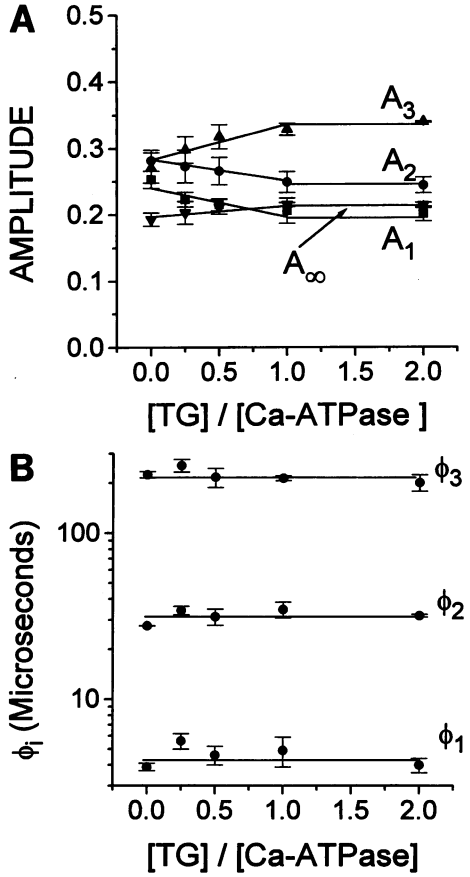


FIGURE 3 (*A*) The amplitudes (A_i) obtained from fitting the TPA curves of ErITC-SR (Fig. 2) to Eq. 2, which models the TPA decay as a sum of three exponentials plus a background term. Addition of TG causes an increase in the amplitudes of the slower rotating species (A_3, A_∞) and a decrease in the amplitudes of the faster ones (A_1, A_2). (*B*) The rotational correlation times (ϕ_i) obtained from fitting the TPA curves to Eq. 2. Addition of TG causes no significant change in the correlation times of any of the three components.

The extent of Ca-ATPase aggregation induced by TG can be monitored by following the amplitudes associated with the various rotating species based on curve-fitting. Previous

TABLE 1 The best-fit values from fitting TPA curves to the exponential decay model of Eq. 2

	A_1	ϕ_1	A_2	ϕ_2	A_3	ϕ_3	A_∞
0:1 TG	0.253 ± 0.013	4.0 ± 0.3	0.282 ± 0.016	27.7 ± 0.3	0.271 ± 0.023	226 ± 10	0.193 ± 0.010
0.25:1 TG	0.223 ± 0.011	5.4 ± 0.3	0.273 ± 0.025	40.0 ± 1.6	0.298 ± 0.020	255 ± 21	0.203 ± 0.017
0.5:1 TG	0.215 ± 0.009	4.4 ± 0.3	0.266 ± 0.021	31.1 ± 3.0	0.318 ± 0.018	217 ± 27	0.212 ± 0.011
1:1 TG	0.206 ± 0.019	4.5 ± 0.5	0.249 ± 0.016	34.1 ± 3.2	0.329 ± 0.009	213.3 ± 7	0.215 ± 0.003
2:1 TG	0.201 ± 0.011	3.6 ± 0.3	0.244 ± 0.012	31.7 ± 1.4	0.340 ± 0.001	202 ± 23	0.214 ± 0.004

These entries are the average and SD obtained after separately fitting the TPA curves from three different samples for each amount of TG. A_i is the normalized amplitude of the ith component, and ϕ_i is the rotational correlation time in μs.

TABLE 2 The best-fit values from fitting the TPA curves to the uniaxial rotation model

	f_1	D_1	f_2	D_2	f_3	D_3	f_i	θ_c	θ_a	ϕ_{ca}	θ_c
0:1 TG	0.210 ± 0.012	178000 ± 8000	0.350 ± 0.026	31800 ± 700	0.400 ± 0.022	4000 ± 200	0.040 ± 0.010	95.8 ± 0.4	63.3 ± 0.1	20.0 ± 0.4	33.5 ± 0.6
0.25:1 TG	0.209 ± 0.005	151000 ± 7000	0.332 ± 0.026	26900 ± 1200	0.414 ± 0.015	3600 ± 200	0.044 ± 0.016	95.5 ± 0.5	63.2 ± 0.2	20.1 ± 0.3	33.8 ± 0.6
0.5:1 TG	0.199 ± 0.014	170000 ± 3000	0.323 ± 0.022	28800 ± 2000	0.424 ± 0.013	4000 ± 400	0.052 ± 0.016	96.1 ± 0.5	63.4 ± 0.2	19.7 ± 0.4	32.7 ± 0.8
1:1 TG	0.175 ± 0.010	157000 ± 19000	0.314 ± 0.013	26000 ± 2300	0.446 ± 0.011	3900 ± 200	0.063 ± 0.009	96.8 ± 0.6	63.5 ± 0.1	19.7 ± 0.5	32.0 ± 0.8
2:1 TG	0.170 ± 0.015	173000 ± 13000	0.310 ± 0.013	27300 ± 300	0.455 ± 0.009	4100 ± 300	0.063 ± 0.004	96.2 ± 0.5	63.5 ± 0.1	19.9 ± 0.2	32.7 ± 0.4

Values were obtained by calculating the average and SDs of fits of at least three separate samples for each amount of TG. D_i is in units of s^{-1}, and angles are in degrees.

studies in this laboratory have fit the curves to a sum of three exponentially decaying terms plus a constant background term (Eq. 2), which is a close approximation to the complete expression describing uniaxial rotation for this case. Table 1 lists the best fit values of the parameters obtained after fitting the data in Fig. 2 to Eq. 2.

These data show that the addition of TG to the Ca-ATPase decreased the amplitudes A_1 and A_2 associated with the rapidly rotating species (short correlation times) and increased the amplitudes A_3 and A_∞ associated with the more slowly rotating ones. There is no significant change in the rotational correlation times themselves. These trends are clarified in Fig. 3 *A* and *B*, which show the effect of TG on the amplitudes and correlation times.

Some of the TPA curves showed a small change in the value of A_{∞_0}. Such a change could be caused by the formation of more immobile species ($f_I > 0$ in Eq. 2) or by a change in the probe orientation relative to the membrane. To investigate the possibility of a probe orientation change, we also fit the TPA curves of the Ca-ATPase in the presence of TG to the function that more exactly describes the anisotropy of uniaxially rotating species (Eqs. 5–7). This function includes explicitly the effects of probe orientation on the preexponential amplitudes (a_1 and a_2 in Eq. 7) as well as on the constant term (a_3 in Eq. 7), and it separates these effects from changes in the mole fractions f_i (Eq. 5). The results of this fit are shown in Table 2.

There is some ambiguity in the angle values listed in Table 2. Because θ_e and θ_a appear symmetrically in Eqs. 7a–c, there is no way for the equation to distinguish between the two. We have arbitrarily chosen θ_e to be the larger of these two angles. Additionally, the fitting function cannot distinguish between the two values of ϕ_{ea} between 0° and 360° that have the same cosine value. Here we have reported the lower value of ϕ_{ea}.

The results from Table 2 have been plotted in Fig. 4 *A–C* for greater clarity. The results of fitting with the more exact equation are similar to those obtained from fitting with the approximate Eq. 2. Upon addition of TG, there is a population shift from the rapidly rotating species f_1 and f_2 to the more slowly rotating ones f_3 and f_I (Fig. 4 *A*). Significantly, there is no change in the rotational diffusion coefficients or in the probe orientation angles (Fig. 4, *B* and *C*). This strongly supports the conclusion that formation of larger oligomers is the main cause of the change in the TPA curve upon addition of TG.

Because it has been proposed that TG stabilizes the Ca-ATPase in the E2 state, we sought to study further the structural basis of the aggregation induced by TG. Fig. 5 shows the effect of preincubating the ATPase in the presence of 1 mM free calcium for 15 min before the addition of TG, a condition known to stabilize the E1 conformation. This Ca preincubation decreases the effect of TG on the anisotropy decay (Fig. 5). The addition of 1 mM Ca after 15 min incubation in TG, however, was unable to reverse the changes in the TPA curve caused by the inhibitor, indicating that TG binds very tightly to the enzyme once the complex has been formed. This is consistent with reports that addition of Ca can

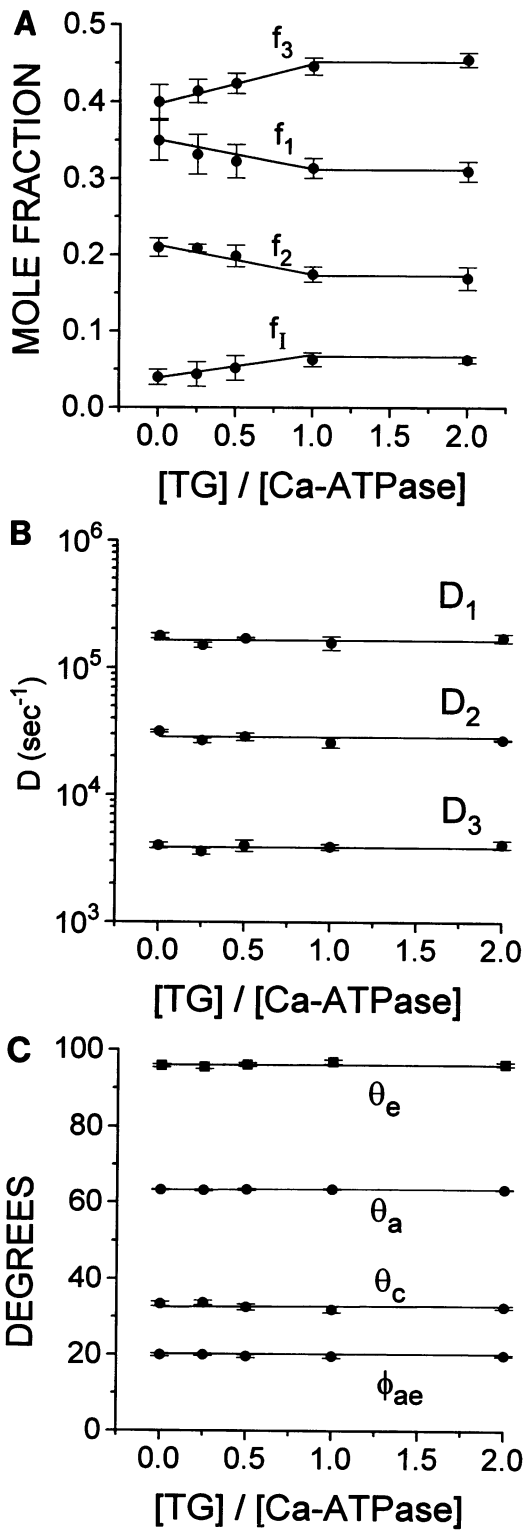


FIGURE 4 The parameters obtained from fitting the TPA curves to Eqs. 5–7, which describe the uniaxial rotation of three species in a membrane. (*A*) The mole fractions. As with the exponential model (Fig. 3 *A*), addition of TG causes a shift in the population distribution from rapidly rotating species (f_1 and f_2) to more slowly rotating ones (f_3 and f_I). (*B*) The rotational diffusion coefficients. TG causes no significant change in this quantity for any of the species. (*C*) The probe orientation angles defined in Fig. 1, and the cone angle for fast wobble motion of the probe relative to the protein. As with the diffusion coefficients, addition of TG has no significant effect on any of these probe angles.

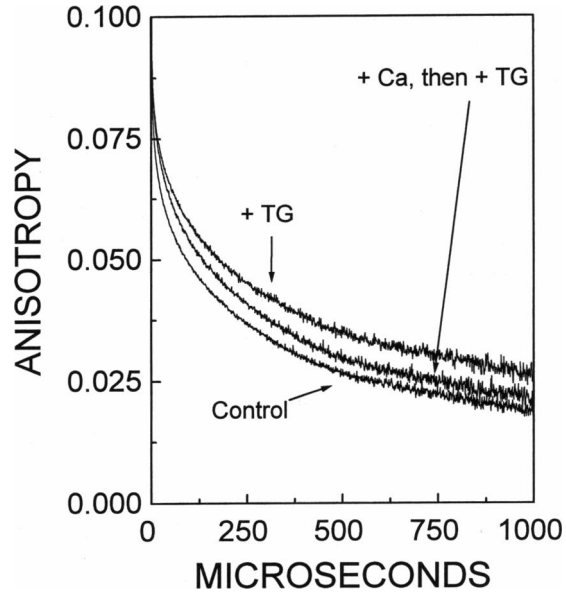


FIGURE 5 The TPA curves of ErITC-Ca-ATPase in the presence of 7 μM TG, and in the presence of 1 mM Ca followed by 7 μM TG. Ca prevents the TG-induced aggregation when Ca is added first, but not when Ca is added second. This TPA curve is not shown, because it completely overlaps the curve with TG alone.

protect against but not reverse the TG-induced inhibition (Sagara et al., 1992b).

DISCUSSION

In the absence of inhibitors, ErITC-labeled Ca-ATPase shows a TPA decay that is well modeled as the decay of three species rotating uniaxially in a membrane, with a constant factor that accounts for fast, restricted rotation of the probe with respect to the proteins. The addition of TG, which strongly inhibits the Ca-ATPase, slows the apparent rate of TPA decay. The Saffman-Delbrück theory (Eq. 11) shows that a change in the rotational diffusion coefficient for a given species can be due to a change in either the membrane fluidity (ruled out for TG by EPR measurements with lipid spin labels) or in the cross sectional area of the protein in the membrane (πa^2). A change in the effective cross section could be caused by a substantial structural change of a given species or by a change in the relative populations f_i in a sample containing a distribution of oligomeric species (Eq. 5). The TPA decay can be affected not only by a change in the rotational diffusion coefficients (or their population distribution), but also by a change in the angles θ_a, θ_e, or ϕ_{ae} (Fig. 1, Eq. 7). Finally, the TPA decays may be changing because TG is acting directly on the probe molecule and disturbing it somehow. Each of these processes must be evaluated for its possible contribution to the change in the TPA decay.

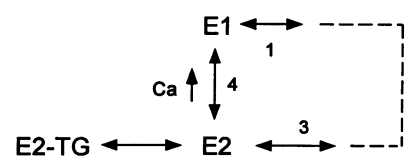
TG induces self-association of Ca-ATPase

We first considered the possibility that TG was interacting directly with the probe molecule to change its TPA decay. The phosphorescence quantum yield, which affects the emission intensity and lifetime, is extremely sensitive to changes in the polarity and fluidity of the immediate environment of the probe. There were no significant changes in the lifetimes or intensities of the phosphorescence decay upon addition of the inhibitor, so it is unlikely that TG interacts directly with the erythrosin. Other spectroscopic quantities whose behavior in the presence of the inhibitor did not change include the angle δ between the probe absorption and emission dipoles (as evidenced by the lack of $r(0)$ change, Eq. 8) and the phosphorescence emission spectrum (data not shown). Thus, the effect of TG on the TPA data is not due to a direct inhibitor-probe interaction.

The next explanation considered for the change in the TPA curve was a change in probe orientation relative to the protein (e.g., due to a protein conformational change upon inhibitor binding). For the approximate fitting function of Eq. 2, the only angle-dependent terms are A_∞ and $r(0)$. The lack of large TG-induced changes in these observed parameters makes angle changes highly unlikely. However, in light of the subtle effects of TG on the overall anisotropy decay, and the occurrence in some of the curves of a small shift in r_∞, it was necessary to carry out a more rigorous analysis of possible angle changes by using Eqs. 5–7, which also include the angular dependencies of the two preexponential factors, a_1 and a_2 (Eqs. 7a and 7b). Equations 5–7 can distinguish much more rigorously between probe angle changes and a redistribution among populations with different rotational rates. The results of this fitting procedure (Table 2 and Fig. 4) show that there are no significant probe angle changes and support firmly the conclusion that the primary effect of TG on the TPA curves is due to a shift in the population from rapidly rotating species to more slowly rotating ones.

Ca can reduce TG effects, but not reverse them

Although the presence of TG caused aggregation of the Ca-ATPase, preincubation of the Ca-ATPase with millimolar concentrations of calcium before the addition of TG substantially reduced the amount of aggregation seen. However, when this calcium was added after the addition of the TG, it did not reverse the aggregation produced. These aggregation effects correlate well with the calcium-dependent inhibition of the Ca-ATPase by TG, as reported by Sagara et al. (1992b), who found that high calcium partially protects the enzyme from TG-induced inactivation by shifting the E2/E1 equilibrium away from the TG-receptive E2 form toward E1 (Scheme 2). The failure of calcium to reverse the effects of



Scheme 2

TG is presumably due to the extremely slow dissociation of TG from the Ca-ATPase.

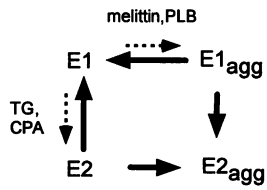
Our finding that calcium protects against TG-induced enzyme aggregation (Fig. 5) correlates our physical data with this kinetic model and suggests that stabilization of the enzyme in the E2 form promotes ATPase aggregation. This is supported by Sagara et al. (1992b), who also reported that calcium is less effective at protecting the Ca-ATPase at reduced temperature, which is known to favor the formation of E2 (de Meis, 1988) and enzyme aggregation (Birmachu and Thomas, 1990; Thomas and Karon, 1994).

Although our results show Ca to be an effective antagonist of TG-induced aggregation, Stokes and Lacapère (1994) have recently shown that millimolar levels of Ca cannot prevent TG-induced formation of tubular Ca-ATPase crystals in the presence of vanadate. The TPA decays described in our report were taken after only 15 min incubation in TG, whereas the crystallization experiments took many days. We also made a number of measurements after several hours incubation with TG, with variable results. In some cases, the aggregation was much greater after several hours than after 15 min, which is consistent with the slow formation of two-dimensional crystals, even without addition of vanadate.

Aggregation correlates with inhibition

Previous reports have shown consistently that a substance or condition that promotes the formation of larger oligomers of the Ca-ATPase also inhibits it, whereas formation of smaller oligomers produces activation (reviewed by Thomas and Mahaney, 1993; Thomas and Karon, 1994). The previously studied effectors, such as peptides (Mahaney and Thomas, 1991; Voss et al., 1991, 1994) and anesthetics (Karon and Thomas, 1993; Kutchai et al., 1994) were less specific than TG for the Ca-ATPase, so it remained a possibility that the aggregation was merely a side effect and not necessarily related directly to the inhibition. A clue about the mechanism of aggregation-induced inhibition has been provided by studying the calcium dependence of this inhibition and the accompanying Ca-ATPase fluorescence changes. These studies on melittin in skeletal SR (Voss et al., 1991, 1995) and phospholamban in cardiac SR (Voss, 1993; Voss et al., 1994) suggest that these inhibitors not only aggregate the Ca-ATPase but also shift its conformational equilibrium from E1 to E2. This raises the possibility that the primary effect of these and other inhibitors is to stabilize E2, which then causes protein self-association. The high affinity and specificity of TG for the Ca-ATPase, along with its striking stabilization of E2, makes it a more incisive probe of the aggregation-inhibition hypothesis. The TPA data show that aggregation does indeed occur, but to a smaller degree than that due to other inhibitors studied. Another useful compound to show this effect is cyclopiazonic acid (CPA), which is also a specific inhibitor of the Ca-ATPase (Seidler et al., 1989; Karon et al., 1994). When ErITC-labeled Ca-ATPase was exposed to CPA, similar changes in the TPA curves and activity were seen as upon addition of TG (Mersol et al., 1993). We propose that both TG and CPA inhibit, as indicated in Scheme 2, by stabilizing the E2 form of the enzyme, which then forms larger oligomers (which E2 is more prone to do than the E1 form).

The stabilization of E2, however, cannot explain the much larger aggregation effects caused by phospholamban (Voss et al., 1994) and by melittin (Voss et al., 1991, 1995). Therefore, we propose that the above compounds represent two classes of inhibitors, which can be explained in a single scheme illustrating how the E1-E2 equilibrium is coupled to pump aggregation (Scheme 3). The solid arrows represent



Scheme 3

the physiologically favored direction of equilibrium at micromolar Ca^{2+}, where the nonaggregated E1 state (*upper left*) predominates. The dashed arrows indicate how these equilibria are perturbed by the two classes of inhibitors. Toxins such as TG and CPA push the Ca-ATPase from E1 towards E2, which then aggregates to form $E2_{agg}$, whereas peptides such as melittin form aggregates of the Ca-ATPase ($E1_{agg}$), which then favors conversion to the $E2_{agg}$ conformation. The clearest example of a physiological role for this inhibitory aggregation is in cardiac SR, in which the Ca-ATPase is regulated by the endogenous amphipathic peptide, phospholamban (PLB). Voss et al. (1994) have shown that the phosphorylation of phospholamban reverses the aggregation and activates the Ca-ATPase, with the primary effects on both occurring at low Ca^{2+}, where E2 has its maximum stability (see Scheme 1).

CONCLUSIONS

The Ca-ATPase-specific inhibitor thapsigargin decreases the apparent rate of TPA decay of the ErITC-labeled pump. We analyzed these data with a refined theoretical model, which treats rigorously the biexponential decays due to several different oligomeric species, including independently the effects of probe orientation and large-scale aggregation. We conclude that the effects of TG on the TPA decay curves are due to a shift in the equilibrium toward larger oligomeric species, rather than to changes in the probe orientation or other effects. The TG:Ca-ATPase stoichiometry and the calcium dependence of this TG-dependent aggregation correlates precisely with Ca-ATPase activity, suggesting that TG inhibits activity by stabilizing the calcium-free (E2) form of the Ca-ATPase, which forms larger oligomers and causes a slower average rate of decay in the TPA curve. Comparison with the results of studies on many other types of inhibitors of the Ca-ATPase show that inhibition is coupled to a change in the oligomeric state of the enzyme, usually aggregation. Such coupling is known to exist for many soluble enzymes (Traut, 1994). These results suggest that the oligomeric state of the Ca-ATPase

plays an essential role in its mechanism of regulation, and we propose that protein self-association is an important regulatory mechanism for membrane proteins in general.

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