ACTA, a fluorescent analogue of thapsigargin, is a potent inhibitor and a conformational probe of skeletal muscle Ca^{2+}-ATPase

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Abstract Thapsigargin is a highly potent and selective inhibitor of sarco-endoplasmic reticulum (SERCA) family of Ca^{2+}-ATPases and a useful tool in research concerning the function of intracellular Ca^{2+} stores. We describe here a novel fluorescent derivative (8-*O*-(4-aminocinnamoyl)-8-*O*-debutanoylthapsigargin, termed ACTA) of this compound, acting as a Ca^{2+}-ATPase inhibitor with a potency approaching that of thapsigargin. Binding of ACTA to the skeletal muscle sarcoplasmic reticulum vesicles results in a strong fluorescence enhancement, approximately 66% of which depends on ACTA association with Ca^{2+}-ATPase. This specific component of ACTA fluorescence is sensitive to the E_1-E_2 conformational equilibrium of the pump. The combined properties of high potency and binding-dependent fluorescence suggest ACTA to be a useful probe for a range of studies involving the SERCA class of ATPases.

Key words: Ca^{2+}-ATPase; Sarco-endoplasmic reticulum Ca^{2+}-ATPase pump; Calcium; Fluorescence; Thapsigargin

1. Introduction

Maintenance of a large gradient between a low cytosolic and a high lumenal Ca^{2+} concentration in the endoplasmic reticulum (ER) is essential for generation of Ca^{2+} signals in eukaryotic cells [1]. The concentration of Ca^{2+} within the ER also influences protein synthesis [2], as well as processing and maturation of the proteins translocating into the ER lumen [3]. Free Ca^{2+} concentrations within the ER are kept at the level of several hundred micromolar to millimolar [3,4] by the action of Ca^{2+}-ATPases. The sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) family of these enzymes is well recognized today, comprising homologous products of three genes [5]. One aspect of the high degree of structural similarity between the SERCA pumps is their uniform sensitivity to inhibition by thapsigargin [6]. The high potency and selectivity of this compound has made it a widely used tool in pharmacological manipulations of Ca^{2+} content of the stores in a large variety of cells [7].

Development of thapsigargin analogues for fluorescent labeling of Ca^{2+}-ATPases would be of interest for a number of applications, including studies of SERCA pump localization in various ER compartments, screening assays for recombinantly expressed Ca^{2+}-ATPase proteins, or structure-activity studies of Ca^{2+}-ATPase enzymes. Previously described fluorescent derivatives of thapsigargin have shown affinities for Ca^{2+}-ATPase at least 2 orders of magnitude lower than the native molecule [8,9]. Here we describe a highly potent, fluorescent thapsigargin analogue, 8-*O*-(4-aminocinnamoyl)-8-*O*-debutanoylthapsigargin (designated ACTA), and demonstrate the ability of this compound to act as a conformational probe due to a specific enhancement of its fluorescence upon association with the thapsigargin binding site of Ca^{2+}-ATPase.

2. Materials and methods

A detailed description of the synthesis of 8-*O*-(4-aminocinnamoyl)-8-*O*-debutanoylthapsigargin (ACTA) is provided elsewhere [10]. Briefly, thapsigargin was isolated from roots and seeds of *Thapsia garganica* as previously described [11]. 8-*O*-Debutanoylthapsigargin (Fig. 1A) was subjected to a dicyclohexylcarbodiimide-facilitated acylation with 4-*t*-butoxycarbonylaminocinnamic acid to obtain 8-*O*-(4-*t*-butoxycarbonylaminocinnamoyl)-8-*O*-debutanoylthapsigargin. This compound was treated with trifluoroacetic acid for 15 min to obtain ACTA. The structure of ACTA was verified by ^{1}H- and ^{13}C-NMR-spectroscopy, and mass spectrometry confirmed the molecular mass to be 725.

Sarcoplasmic reticulum (SR) vesicles from rabbit fast twitch skeletal muscle (expressing SERCA1 Ca^{2+}-ATPase) were prepared according to De Meis and Hasselbach [12] and stored in *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) 20 mM (pH 7.0), sucrose 300 mM, at −80°C. Ca^{2+}-ATPase accounted for at least 50% of the total protein in this preparation, based on the densitometric scanning of Coomassie Blue-stained sodium dodecyl sulfate (SDS) polyacrylamide gels.

ATPase activity was measured as the ATP-dependent $^{45}Ca^{2+}$ uptake to SR vesicles at 25°C, pH 7.0. 50 μg/ml of protein was used in all uptake experiments except those indicated in the legend to Fig. 1D. Triplicate SR samples were preincubated for 10 min in the uptake medium minus ATP, in the presence of the desired concentrations of thapsigargin or ACTA, added as 100- or 200-fold concentrated stock solutions in dimethyl sulfoxide (DMSO; a corresponding amount of DMSO was present in control incubations). The medium was then completed by adding ATP (or TES for blank determinations) to start the uptake reaction. The complete medium contained in 1.0 ml (concentrations in mM): TES 20; sucrose 60; KCl 110; $MgCl_2$ 5; K^+-oxalate 6; EGTA 1; $^{45}CaCl_2$ 0.2 (about 1 μCi/ml); ATP 2.5 mM. (The calculated concentration of free Ca^{2+} ions was 0.1 μM [13].) The uptake was stopped after 4 min by filtering the mixture through Whatman GF/F filters (presoaked in 0.1% polyethyleneimine to reduce the background radioactivity) on a Brandel Harvester M24 apparatus (SEMAT Technical, UK). The filters were washed immediately in an ice-cold buffer containing (concentrations in mM): Tris-HCl 20 (pH 7.0); NaCl 140; $MgCl_2$ 10; and counted using Opti-Fluor scintillation liquid (Packard Instruments, DK) at 100% efficiency. The time course of the uptake was linear for at least up to 4 min with all the protein concentrations employed.

The IC_{50} or EC_{50} values are means of the numbers obtained from

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Abbreviations: DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; SDS, sodium dodecyl sulfate; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TES, *N*-tris(hydroxymethyl)-methyl-2-aminomethane sulfonic acid

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4-parameter logistic equation fitting of the dose-response data (GRAFIT program, Erithacus Software), with the number of individual experiments indicated in figure legends.

The fluorescence measurements were carried out in a stirred, thermostated cuvette of a Hitachi F-2000 spectrofluorimeter, at 10 nm bandpass setting. Excitation and emission wavelengths of 352 nm and 435 nm, respectively, were used in all measurements shown, except for the emission spectra. The medium in the cuvette (2.5 ml) consisted of (concentrations in mM): TES 20; KCl 110; $MgCl_2$ 5; EGTA 1; SR vesicles at the protein concentration 50 μg/ml. When desired, total amounts of $CaCl_2$ were included in the mixture to obtain the indicated free Ca^{2+} concentrations, calculated according to Föhr et al. [13]. Thapsigargin or ACTA dissolved in DMSO were added to obtain the final concentrations indicated in the figures. The final total DMSO concentration did not exceed 1%. Unless indicated otherwise, the fluorescence measurements were carried out at 25°C, pH 7.0.

Protein concentration was determined by the fluorescamine method [14].

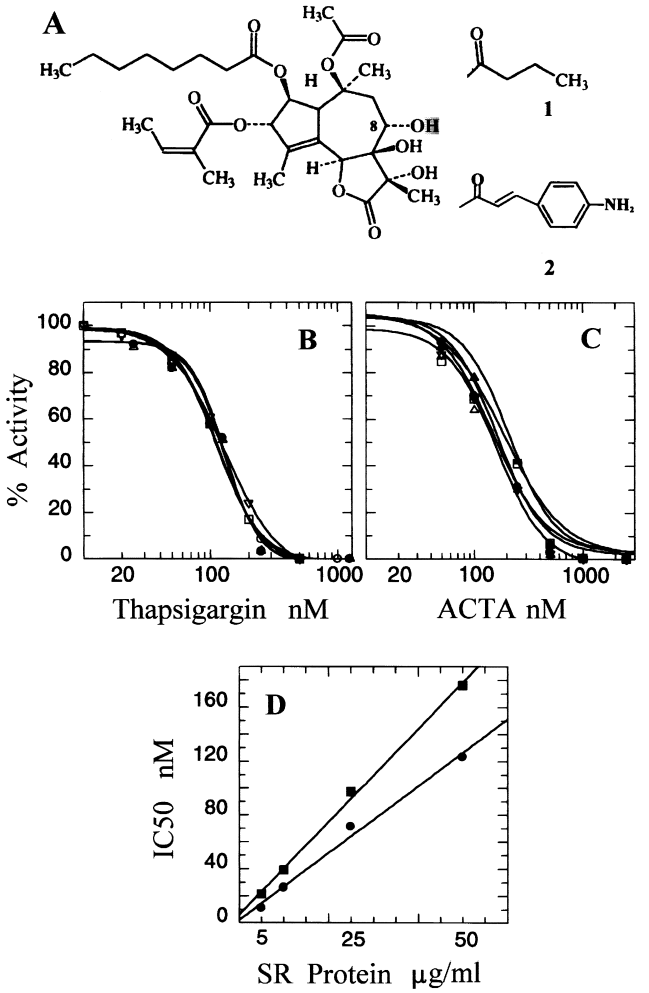

Fig. 1. Comparison of Ca^{2+}-ATPase inhibition due to thapsigargin and ACTA. A: Thapsigargin or ACTA is obtained by substitution of butanoyl (**1**) or 4-aminocinnamoyl (**2**) moiety, respectively, for the hydroxyl group hydrogen (shaded) in the C8-position of the 8-*O*-debutanoylthapsigargin, shown on the left. B: Inhibitor concentration dependence of thapsigargin-mediated inhibition of $^{45}Ca^{2+}$ uptake to SR vesicles. Mean IC_{50} = 123.1 nM, S.E.M. = 3.9. C: Inhibitor concentration dependence of ACTA-mediated inhibition of $^{45}Ca^{2+}$ uptake to SR vesicles. Mean IC_{50} = 167.5 nM, S.E.M. 8.6 (B and C show data and fitted curves from 5 individual experiments). D: Dependence of the apparent potencies (expressed as IC_{50} values) of thapsigargin (●) and ACTA (■) as inhibitors of $^{45}Ca^{2+}$ uptake on the concentration of SR protein.

3. Results and discussion

3.1. ACTA is a potent inhibitor of Ca^{2+}-ATPase

ACTA was obtained by a substitution of a 4-amino-cinnamoyl moiety for the C8 hydroxyl group hydrogen of 8-*O*-debutanoylthapsigargin (Fig. 1A). Fig. 1B and C compare the inhibition obtained with thapsigargin and ACTA, respectively, of the ATP-dependent $^{45}Ca^{2+}$ uptake to rabbit skeletal muscle SR vesicles under identical assay conditions. It is clear that the chemical modification resulted only in a slight increase of the ACTA IC_{50} (167.5 nM, S.E.M. 8.6; $N = 5$), as compared with the IC_{50} for thapsigargin (123.1 nM, S.E.M. 3.9; $N = 5$). It is well established that under the conditions when the pump activity is well expressed, the binding of thapsigargin to Ca^{2+}-ATPase is stoichiometric and dependent on the enzyme concentration [15]. When the protein concentration dependence of the IC_{50} values for thapsigargin-mediated and ACTA-mediated inhibitions of $^{45}Ca^{2+}$ uptake were tested, a linear relationship was obtained for each of the substances (Fig. 1D), with the slope ratio of ACTA to thapsigargin of 1.39. These data show that ACTA is a potent inhibitor of Ca^{2+}-ATPase, and are consistent with the possibility of ACTA-ATPase interaction at the site involved in the thapsigargin-mediated inhibition of the pump.

3.2. The increase of ACTA fluorescence upon binding to Ca^{2+}-ATPase reflects formation of the ACTA-enzyme inhibitory complex

With the emission wavelength set to 450 nm, the excitation spectrum of ACTA in the absence of protein displayed a major peak at 352 nm and a minor one at 245 nm (not shown). A series of emission spectra excited at 352 nm is shown in Fig. 2A. The broad fluorescence peak of ACTA 0.5 μM in the absence of protein showed a maximum at 446 nm (Fig. 2A, trace 1). In the presence of SR vesicles, successive additions of ACTA resulted in a gradual increase of the fluorescence and an apparent shift of the peak to 435 nm (Fig. 2A, traces 3–9). Excitation and emission wavelengths of 352 and 435 nm, respectively, were employed in the remaining experiments. The fluorescence intensity was sensitive to temperature: an increase was observed with decreasing temperature between 37°C and 20°C. This effect of temperature was not investigated any further.

In view of the ability of ACTA to act as an inhibitor of Ca^{2+}-ATPase with a potency approaching that of thapsigargin, it was likely that the increase of ACTA fluorescence in the presence of SR vesicles reflected its association with Ca^{2+}-ATPase at the thapsigargin binding site. To test this assumption, the sarcoplasmic reticulum membranes were preincubated with increasing concentrations of thapsigargin prior to the addition of ACTA (Fig. 2B). A thapsigargin dose-dependent inhibition of the fluorescence enhancement was observed, with a residual value reached at 1 μM thapsigargin and an IC_{50} = 151.7 nM (S.E.M. 10.2, $N = 5$). This competition by thapsigargin with respect to ACTA binding defined a major component (approximately 66%) of the ACTA fluorescence enhancement which might be termed specific (resulting from ACTA association with Ca^{2+}-ATPase), and a non-specific residual component, reflecting ACTA interaction with other protein and lipid constituents of the sarcoplasmic reticulum vesicles. Fig. 2C shows that the specific component was satu-

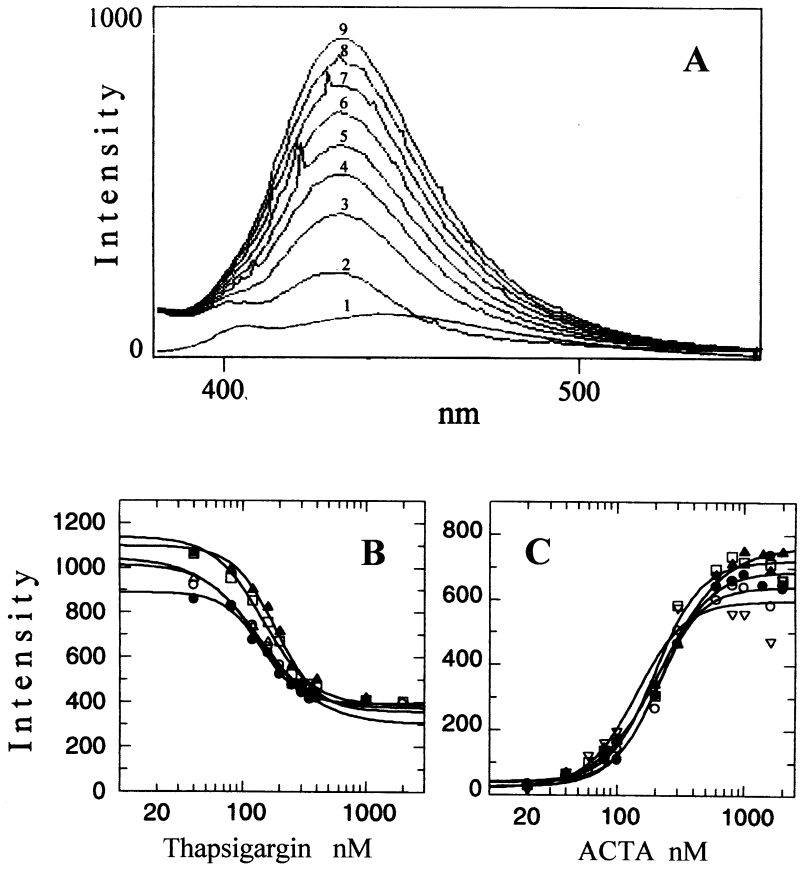

Fig. 2. Increase in ACTA fluorescence upon binding to Ca^{2+}-ATPase. A: Emission spectra excited at 352 nm and obtained in the presence of ACTA 0.5 μM alone (trace 1), SR vesicles alone (trace 2), or SR vesicles in the presence of ACTA concentrations increasing from 50 nM (trace 3) in increments of 25 nM (traces 4–8) to the total of 200 nM (trace 9). B: Concentration dependence of thapsigargin-mediated inhibition of the fluorescence of ACTA 1 μM in the presence of SR vesicles. Mean $IC_{50} = 151.7$ nM (S.E.M. 10.2). C: Dependence of the specific component of ACTA fluorescence enhancement on ACTA concentration. Mean $EC_{50} = 214.2$ nM (S.E.M. 5.7). For the indicated ACTA concentrations in C, points represent differences between pairs of values determined in the absence and presence of 2 μM thapsigargin. In B and C, thapsigargin was added 3 min before ACTA; data and fitted curves from 5 individual experiments are shown.

rated at about 1 μM ACTA, with an $EC_{50} = 214.2$ nM (S.E.M. 5.7, $N = 5$).

In summary, the inhibition of Ca^{2+}-ATPase by ACTA with a potency close to that of thapsigargin (Fig. 1B and C), the competitive effect of thapsigargin on the enhancement of ACTA fluorescence with a potency similar to its potency as an inhibitor of Ca^{2+}-ATPase (Figs. 2B and 1B), as well as the similarity of the saturation concentrations of ACTA and thapsigargin in binding (Fig. 2C) and binding inhibition (Fig. 2B), respectively, supported the specific enhancement of ACTA fluorescence being due to ACTA association with the site on Ca^{2+}-ATPase utilized by thapsigargin. Taken together with the finding of the very similar apparent ACTA affinities in the binding and $^{45}Ca^{2+}$ uptake inhibition measurements (Figs. 2C and 1C), these data are also consistent with the inhibitory activity and specific fluorescence change of ACTA representing different manifestations of the formation of the same ACTA-Ca^{2+}-ATPase complex.

3.3. *The sensitivity of ACTA fluorescence to Ca^{2+}-ATPase conformational state*

Additional confirmation of the specific ACTA fluorescence reflecting the interaction of this compound with the thapsigargin-binding site on Ca^{2+}-ATPase was obtained in experiments seeking to demonstrate a dependence of this fluorescence on the conformational equilibrium of the enzyme in the presence of the inhibitor. The rationale behind these experiments was that such dependence would be expected to follow the rules elicited for the association of thapsigargin with the pump. The structural changes of the Ca^{2+}-ATPase involved in thapsigargin binding have been interpreted in terms of the E_1-E_2 conformational model [16,17] and studied in considerable detail [18,19]. These studies have indicated that the enzyme species responsible for the high overall affinity of thapsigargin for Ca^{2+}-ATPase is E_2, i.e. the species unable to bind Ca^{2+} with high affinity. Therefore, if high-affinity ACTA binding similarly was dependent on E_2 conformation, one should be able to manipulate the rate of specific fluorescence enhancement, as well as the level at equilibrium, by changing the fraction of Ca^{2+}-ATPase in the E_2 state.

To test this prediction, a series of decreasing E_2/E_1 conformational ratios was obtained by increasing the concentration of Ca^{2+} in the medium at two different combinations of temperature and pH (Fig. 3A,B). In each case, addition of ACTA produced a fast component of fluorescence increase, beyond the time resolution of our experiments, followed by a slower component rising towards a plateau value. When 2 μM thapsigargin was present in the cuvette prior to the addition of ACTA, the minimal plateau level was obtained, with the rise to this level almost exclusively consisting of the fast compo-

nent. Thus, fluorescence increments above this minimal level specifically reflected ACTA association with Ca^{2+}-ATPase. Best seen in Fig. 3A and C, this specific portion of the traces with no, or the smallest amount (1.2 μM) of free Ca^{2+} present still contained an unresolved fast component, followed by a slower rise. The findings in Fig. 3A and B were that the fraction of the specific fluorescence rise occurring as a fast component, the rate of the slow component, and the plateau level were all diminished upon increasing Ca^{2+} concentrations. These findings may be accounted for with reference to the scheme in Fig. 3D. Following mixing with the SR vesicles, a fast binding of ACTA takes place to the non-ATPase (thapsigargin-insensitive) sites as well as to that fraction of Ca^{2+}-ATPase present in the E_2 conformation. Since in the absence of any added Ca^{2+} this fraction is smaller at 25°C and pH 8.0 than at 37°C and pH 7.0 (Fig. 3B and A, respectively) [18,20,21], a greater portion of the specific fluorescence rise occurs as the fast, temporally unresolved component in Fig. 3A than in Fig. 3B (top trace in each figure). With 1 μM ACTA previously found sufficient for saturation of the specific binding (Fig. 2C), use of 2 μM ACTA in the experiments of Fig. 3A and B led to a rapid depletion of the E_2 species available for the fast binding phase. After this depletion, the slow $E_1 \rightarrow E_2$ transition would become rate-limiting for fur-

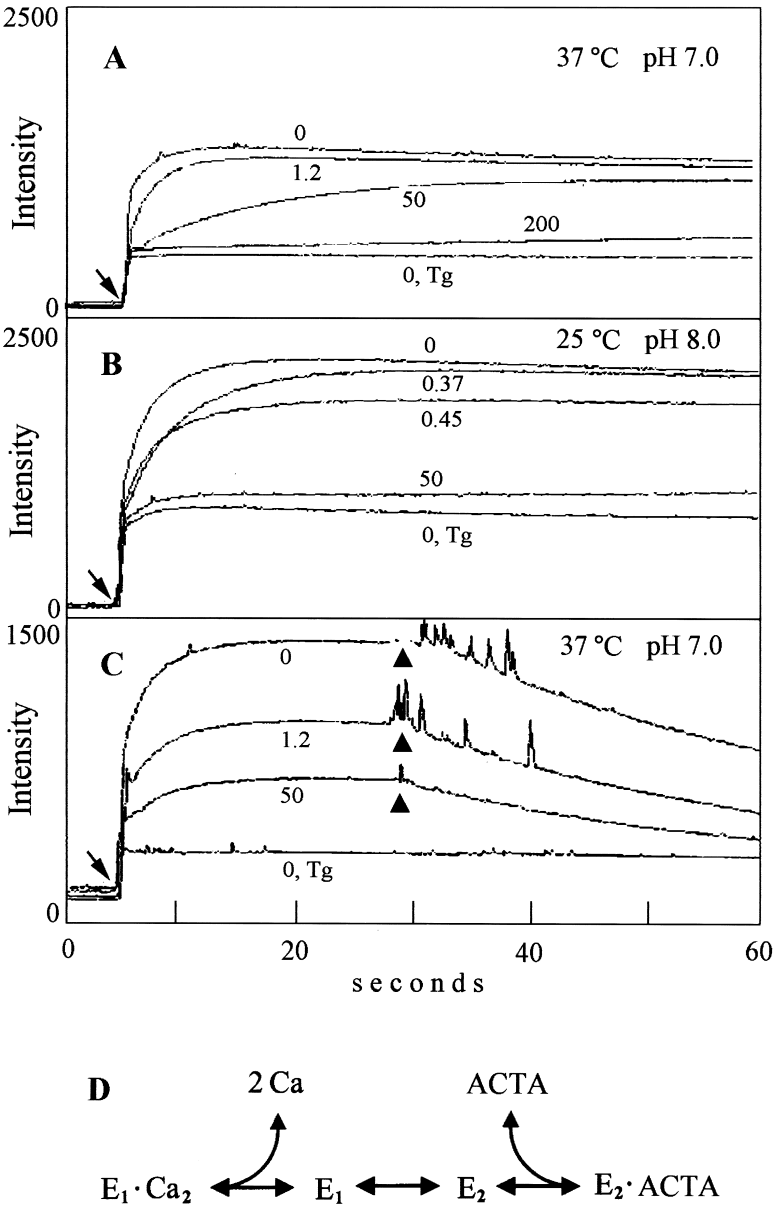

Fig. 3. The sensitivity of ACTA fluorescence to Ca^{2+}-ATPase conformational state. In A, B and C, numbers adjacent to the individual fluorescence records indicate nominal free Ca^{2+} concentrations (μM), calculated from the total added Ca^{2+} as described in Section 2. Arrows indicate addition of ACTA to final concentrations of 2 μM (A and B) or 1 μM (C). Triangles in C indicate addition of thapsigargin to the final concentration 20 μM. In the records marked by 'Tg', thapsigargin (final 10 μM) was added 3 min prior to ACTA. In A, B and C, temperature and pH were as indicated. Each set of records is representative of 5–10 experiments. D: The proposed reaction scheme consistent with the ACTA-Ca^{2+}-ATPase interaction at the thapsigargin-binding site and with the observed sensitivity of the specific ACTA fluorescence to the ATPase conformational equilibrium.

ther ACTA binding and fluorescence increase. Increasing concentrations of Ca^{2+} will be expected to decrease the availability of free E_1 for the conversion to E_2 and to slow it down, as well as to decrease the total amount of E_2-ACTA complex at equilibrium, as indeed observed in Fig. 3A and B. It may also be seen that under the conditions relatively more favorable to the E_2 conformation (i.e. 37°C and pH 7.0 rather than 25°C and pH 8.0), higher Ca^{2+} concentrations were necessary for comparable degrees of inhibition of specific ACTA fluorescence rise, consistent with the decrease in the apparent affinity of Ca^{2+} for the enzyme when the E_2 conformation is favored.

This interpretation of the ACTA binding in terms of the equilibrium between the two distinct conformational states E_1 and E_2 is analogous to the interpretation of the changes in the intrinsic Ca^{2+}-ATPase fluorescence upon binding of thapsigargin presented earlier by Sagara et al. [18]. Most clearly this analogy may be seen by referring to Fig. 7 of their report, in which the various ratios between the fast and the slow components of the intrinsic fluorescence change were interpreted in terms of the shifting equilibrium between the E_2 and E_1 conformational states, the slow component reflecting the rate of isomeric conversion to E_2 driven by thapsigargin binding.

In agreement with the scheme of Fig. 3D, Fig. 3C shows that when ACTA concentration was decreased from 2 to 1 μM, the inhibitory effect of Ca^{2+} on the specific ACTA fluorescence rise was more pronounced (compare with Fig. 3A). Fig. 3C also shows that dissociation of ACTA could be revealed when at the point of binding steady-state, a 20-fold excess of thapsigargin was added to prevent the reassociation of ACTA with the enzyme.

3.4. Concluding remarks

To our knowledge, this is the first report describing a thapsigargin analogue whose fluorescence increases strongly upon its association with the thapsigargin binding site on Ca^{2+}-ATPase. Fluorescent thapsigargin derivatives obtained by replacing the butanoyl moiety with *N*-dansylglycine or eosin in the C8 position of thapsigargin were described earlier [9]. While dansylthapsigargin proved useful for a study of the fluorescence energy transfer between the tryptophanyl residues in the membrane-bound region of Ca^{2+}-ATPase and the enzyme-bound fluorophore [9], the effect of binding on the fluorescence of directly excited dansylthapsigargin was not considered. However, the relatively low potency of dansylthapsigargin as Ca^{2+}-ATPase inhibitor (200-fold less compared to thapsigargin) would suggest that a detection of binding-related changes in its fluorescence might be difficult, given the excess of uncomplexed, fluorescent ligand. In contrast, the high affinity of ACTA for Ca^{2+}-ATPase (Fig. 1C,D, Fig. 2C), along with an approximate 2:1 ratio between the maximal specific and non-specific components of its fluorescence enhancement at the saturating ACTA concentration (Fig. 2B), allowed convenient measurements of the fluorescence changes due to ACTA-ATPase binding.

The SR vesicles from rabbit fast twitch muscle employed here contain SERCA1 pump. Thapsigargin has proved an equally potent inhibitor of SERCA1a, SERCA2a, SERCA2b and SERCA3 isoforms [15], and the region of SERCA1 (S3–M3) implicated in thapsigargin binding [22,23] is highly conserved among SERCA1–3 subtypes [22]. Therefore, it would seem reasonable to expect ACTA to display similar properties when binding to other proteins of the SERCA family.

Our experiments did not directly address the underlying physical basis for the fluorescence behavior of ACTA molecule. Nevertheless, this behavior seems consistent with the available evidence concerning the topology of the Ca^{2+}-ATPase thapsigargin binding site. Bound thapsigargin has been suggested to occupy a position within the membrane at the lipid-protein interface [24], while specifically interacting with the near-membrane portion of the S3 segment [23]. Therefore, ACTA binding at this site might involve a number of microenvironmental effects with a potential to affect the fluorescence yield of its aromatic fluorophore, including effects exerted by site polarity, changes in ligand solvation, or ligand structure geometry [25].

Among the possible future applications of ACTA, its behavior as a conformational probe might be of particular interest in studies of structure-function relationships in Ca^{2+}-ATPase. For instance, in the dissection of functional Ca^{2+}-ATPase domains by directed mutagenesis it is sometimes necessary to differentiate possible displacements of E_1-E_2 equilibrium from other mutation-induced effects [26]. This type of differentiation would be facilitated by the conformational sensitivity of ACTA fluorescence, allowing the conformational information to be obtained in a manner not necessarily dependent on other properties of the studied mutants, e.g. the properties of their phosphorylation reactions.

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