Specific Structural Requirements for the Inhibitory Effect of Thapsigargin on the Ca^{2+}-ATPase SERCA*

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Mutational analysis of amino acid residues lining the thapsigargin (TG) binding cavity at the interface of the membrane surface and cytosolic headpiece was performed in the Ca^{2+}-ATPase (SERCA-1). Specific mutations such as F256V, I765A, and Y837A reduce not only the apparent affinity of the ATPase for TG but also the maximal inhibitory effect. The effect of mutations is dependent on the type and size of the substitute side chain, indicating that hydrophobic partitioning of TG and complementary molecular shapes are involved not only in binding but also in the inhibitory mechanism. A major factor determining the inhibitory effect of bound TG is its interference with conformational changes that are required for the progress of the ATPase cycle. Most prominent and specific is the TG interference with a wide displacement of the Phe-256 side chain that is associated with the E2 to E1Ca^{2+} transition. The specificity of the TG inhibitory mechanism is emphasized by the finding that the F256V mutation does not interfere at all with the effect of 2,5-di-((butyl)-hydroquinone, which is another SERCA inhibitor bound by hydrophobic partitioning. The specificity of the inhibitory mechanism is also emphasized by the observation that within the concentration range producing total inhibition of wild-type SERCA-1, TG produces a 4-fold stimulation of the P-glycoprotein (multidrug transporter) ATPase.

Sesquiterpene lactone thapsigargin (TG), derived from the plant *Thapsia garganica* (1, 2), is a potent and highly specific inhibitor of the endo- (3) and sarcoplasmic reticulum (4–6) Ca^{2+}-ATPases (SERCA). Because of the role of intracellular Ca^{2+} stores in control and regulation of numerous cellular functions (7, 8), TG is used extensively as a pharmacological tool in physiological experimentation (9, 10). Furthermore, TG is being considered for targeted cancer treatment (11) because of its inhibitory effect on cell proliferation (12).

Inhibition of Ca^{2+}-ATPase is observed at subnanomolar concentrations of TG and is attributed to drug binding to the enzyme with a 1:1 molar ratio (4). TG induces stabilization of the ATPase protein in a state similar to that of the Ca^{2+}-free enzyme (i.e. E2), facilitating formation of ordered arrays (13) for structural studies by electron microscopy and x-ray diffraction (14, 15).

Spectroscopic (16), photolabeling (17), chimerization (18), and mutational studies (19, 20) suggested that the TG binding site resides near the M3 segment at the cytosolic membrane surface. TG was then clearly identified by diffraction studies (15) in a cavity delimited by the M3, M5, and M7 transmembrane helices near the cytosolic surface of the membrane (Fig. 1). We report here a mutational study, including construction and characterization of 20 mutations within the region surrounding the TG binding pocket. The aim of this study was to evaluate specific structural features that may be involved in determining the ATPase affinity for TG and its inhibitory mechanism. The specificity of the TG inhibitory mechanism was further investigated with comparative experiments on the SERCA inhibitor 2,5-di-((butyl)-hydroxybenzene (DBHQ) (22, 23) and on the ATPase of the P-glycoprotein drug transporter (24, 25).

**EXPERIMENTAL PROCEDURES**

**Preparation of Membrane Vesicles**—Native sarcoplasmic reticulum vesicles were obtained from rabbit skeletal muscle as described previously (26).

**PCR Mutagenesis and Protein Expression**—The chicken fast muscle SERCA-1 cDNA (27) was subcloned into the SV40-pAdlox vector for site-directed mutagenesis. Primers of 20–30 bp in length were synthesized for each individual mutation. The primers were utilized to hybridize DNA sequences internal to the flanking primers and were used for PCR mutagenesis by the overlap extension method as described by Ho et al. (28). Briefly, two overlapping fragments containing the mismatched bases of the targeted sequence were amplified in separate PCRs. The reaction products were then mixed and amplified by PCR using both flanking primers. The mutant cassette was then exchanged with the corresponding cassette of wild-type cDNA in SV40-pAdlox. After DNA sequencing, the mutated cDNA was transfected into COS-1 cells for overexpression of protein under control of the SV40 promoter. COS-1 cell cultures and transfection methods were described by Sumbilla et al. (29).

**Microsomal Preparation and Immunodetection of Expressed Protein**—The microsomal fraction of transfected COS-1 cells was obtained by differential centrifugation of homogenized cells (29). Immunodetection of expressed ATPase in the microsomal fraction was obtained by Western blotting using 9E10 monoclonal antibody against the c-myc tag of SERCA-1. WT human P-glycoprotein was expressed in yeast, purified, and reconstituted in liposomes as described by Figler et al. (30).

**Synthesis of TG Analog Debutanoyl Thapsigargin**—TG (2.0 mg = 3.1 μmol) was dissolved in methanol and 10 μl of triethylamine and then incubated with stirring at room temperature for about 5 h in a small capped tube to carry selective cleavage of the ester bond at C-8. The reaction was followed by high pressure liquid chromatography analysis as described by Hua and Inesi (17). The yield of the product was ~90%.
**RESULTS**

**Transgenic Expression of WT and Mutant SERCA—Approximately 10% of the COS-1 cells transfected under our conditions exhibited exogenous SERCA gene expression and targeted the Ca\(^{2+}\) ATPase to the endoplasmic reticulum, as shown by immunofluorescent staining and microscopy (36). For the experiments reported here, we expressed the WT enzyme and 20 mutants resulting from single or double mutations of 10 amino acid residues near the TG binding site at the interface of the membrane-bound region with the headpiece of the ATPase. In designing the mutational work, we selected amino acid residues adjacent to the TG ring structure.

The expression levels of transgenic SERCA are variable, partly because of the efficiency of transfection and partly because of the presence of mutations. The levels were determined by Western blots and densitometry and compared with a standard preparation of the WT enzyme (Fig. 3). The average values listed on Table I were obtained from various transfections and therefore reflect mostly the specific effects of the mutations on the levels of transgenic protein.

Most of the mutants were expressed with lower efficiency than the WT ATPase, such as L253A, F256L or F256V, G257F, Q259E or Q259F, and I761A. Most notably, the F834D mutation strongly reduced expression, whereas F834A and F834S mutations allowed significant expression levels. It is apparent that the presence of charged residues in critical locations interferes with proper folding of the nascent polypeptide chain even in the presence of normal transcription levels (36). Interestingly, all of the expressed mutants retained relatively high ATPase activity with some reduction noted for the Q257K and I765G mutants. There was no correlation between the expression levels and the ATPase activity of the mutants.

**ATPase Activity and Effects of Mutations—**The total ATPase activity of microsomal preparations includes a Ca\(^{2+}\)-independent component that we subtracted from the total to obtain the specific Ca\(^{2+}\)-dependent ATPase. It should be pointed out that the activity of all mutants was corrected to reflect the SERCA content of the microsomes used in the assays with reference to the same preparation of the WT ATPase. No significant levels of TG-inhibited Ca\(^{2+}\) ATPase activity were detected in non-or sham-transfected COS-1 cells.

It is shown in Fig. 4 that the WT Ca\(^{2+}\) ATPase is inhibited within the nanomolar concentration range of TG, and total inhibition of the Ca\(^{2+}\)-dependent ATPase is observed in the presence of 1 mM TG. This effective concentration range of TG remains unchanged for certain mutants, such as F834A. On the other hand, ATPase inhibition requires higher TG concentrations when other mutants are used, such as F256V. In addition, the maximal inhibition that can be obtained by increasing the TG concentration is significantly reduced (Fig. 4A). Furthermore, for a given amino acid, the inhibitory effect of TG depends on the type of mutation, i.e. F256V as compared with F256L (Fig. 4B). The effects of various mutations on the ATPase sensitivity to TG are shown in Table I, where the TG concentrations producing a 50% inhibition is defined as \(K_i\). Pronounced effects on \(K_i\) were observed for the F256V and I765G mutations, whereas lower but significant effects were produced by the Y837A, I765A, Y837F, Q259K, and F256L mutations.

**WT and F256L Affinity for a TG Analog—**In all of the experiments reported above the inhibitory effect of TG is given as a function of "total added" TG. However, an evaluation of the actual \(K_i\) would require knowledge of the "free" TG concentration. In fact, because of the high affinity and the low concentrations of TG relative to the ATPase, the residual free TG concentration in solution mixtures may be significantly lower than the total concentration thereby yielding an underestimate of the mutational effects on the affinity of the inhibitor for the enzyme. As a case in point, we tested the effect of

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\(^{a}\) Y. Sugita, M. Ikeguchi, and C. Toyoshima, unpublished work.
DBTG on a mutant (F256L) on which the TG concentration curve is only moderately displaced relative to the WT enzyme. DBTG is a debutanoyl derivative of TG exhibiting lower affinity for SERCA (17). In this case the free drug concentration is approximately equal to the total concentration because the ATPase in the reaction mixture is 0.5 nM, and the inhibitory effect of DBTG on WT ATPase is observed within the 5–500 nM range. As shown in Fig. 5, the drug concentrations required to obtain inhibition of the F256L mutant are two orders of magnitude higher when DBTG is used instead of TG. This suggests that the experiments with TG may in fact underestimate the mutational effects on the affinity of the inhibitor for the ATPase.

TG Interferes with Conformational Changes—To gain a better understanding of the mechanism of inhibition, we considered that TG wedged at the interface of the M3, M5, and M7 transmembrane segments may interfere with structural transitions that are required for the progress of the ATPase cycle. An assessment of these transitions can be conveniently derived from observation of the pattern of ATPase digestion by protein-

![Thapsigargin (TG), debutanoyl thapsigargin (DBTG) and 2,5-di-(t-butyl)-hydroquinone (DBHQ).](image)

![Examples of Western blot analyses of transgenic SERCA expression in COS-1 cells.](image)

**TABLE I** Effects of mutations on the Inhibitory effect of TG

| Sample | Expression | Ca\(^{2+}\) ATPase activity | \(K_i^a\) |
|--------|------------|----------------------------|--------|
|       |            | nmol of Pi/mg of protein/min | nM     |
| WT    | 1          | 150                        | 1.44 ± 0.77 |
| K252A | 1.000      | 169.0 ± 13.0               | 2.35 ± 1.02 |
| L253A | 0.122      | 147.0 ± 29.5               | 1.26 ± 0.21 |
| F256V | 0.318      | 186.0 ± 29.2               | 46.63 ± 25.23 |
| F256L | 0.313      | 161.3 ± 5.0                | 3.57 ± 2.20 |
| G257K | 0.552      | 61.5 ± 11.6                | 1.44 ± 0.40 |
| G257E | 0.516      | 97.5 ± 1.2                 | 1.38 ± 0.34 |
| G257F | 0.258      | 159.0 ± 8.5                | 2.23 ± 0.89 |
| Q259K | 0.981      | 100.5 ± 8.8                | 3.26 ± 1.37 |
| Q259E | 0.318      | 168.0 ± 19.5               | 0.26 ± 0.09 |
| Q259F | 0.318      | 175.5 ± 22.8               | 1.25 ± 0.51 |
| Q259A | 1.014      | 120.0 ± 7.3                | 2.66 ± 0.75 |
| I761A | 0.331      | 91.5 ± 10.6                | 0.46 ± 0.22 |
| I765G | 0.381      | 66.0 ± 10.0                | 15.81 ± 11.59 |
| I765A | 0.396      | 139.5 ± 6.7                | 7.67 ± 7.65 |
| I829A | 0.777      | 118.5 ± 3.0                | 2.88 ± 0.95 |
| F834S | 0.678      | 127.5 ± 30.1               | 1.82 ± 1.29 |
| F834F | 0.519      | 139.5 ± 8.2                | 5.11 ± 3.06 |
| F834D | 0          | NA\(^b\)                    | NA     |
| Y837A | 0.291      | 141.0 ± 3.1                | 6.17 ± 7.22 |
| Y837F | 0.462      | 145.5 ± 7.3                | 3.25 ± 1.61 |

\(^a\) \(K_i\), TG concentration to generate 50% inhibition.

\(^b\) NA, not applicable.
ase K (37). In fact, electrophoretic analysis of fragments produced by digestion in the absence of Ca\(^{2+}\)/H11001 yields a light 95-kDa band (Leu-119 site) and a heavier 83-kDa band (Thr-242 site). In the presence of Ca\(^{2+}\)/H11001, the digestion proceeds faster, but the 95-kDa band is not produced. This difference in digestion pattern is attributed to a Ca\(^{2+}\)/H11001-dependent conformational transition (E2 to E1/Ca\(^{2+}\)), which involves rotation of the A domain with a consequent influence on the digestion sites within the loop connecting the M2 segment to the A domain (15). It is shown in Fig. 6 that TG prevents such an effect of Ca\(^{2+}\), a st the 95-kDa band is still produced in the presence of Ca\(^{2+}\), and the ATPase band remains more intense. Furthermore, a slight effect of TG is noted even in the absence of Ca\(^{2+}\), indicating that the equilibrium between E1 and E2 states is not shifted by the mutations per se thus excluding that such a shift could reduce the apparent affinity of the F256 and I765 for TG.

Pertinent to the resistance of mutants to inhibition, we found that TG fails to prevent the effect of Ca\(^{2+}\) even in the presence of TG (Fig. 6). On the other hand, the F834A mutant, which does not interfere with the TG effect, yields a digestion pattern nearly identical to that obtained with WT enzyme (Fig. 6). This provides additional evidence indicating that the TG inhibition mechanism is related to interference with conformational changes that are required for the catalytic activity of the Ca\(^{2+}\) ATPase.

It is noteworthy that the distinct patterns of proteolysis obtained in the presence of Ca\(^{2+}\) and in the absence of TG (lane 2 in each panel), or the absence of both Ca\(^{2+}\) and TG (lane 4 in each panel), are identical in WT and mutants. This indicates that the equilibrium between E1 and E2 states is not shifted by the mutations per se thus excluding that such a shift could reduce the apparent affinity of the F256 and I765 for TG.

**Specificity of the Inhibitory Mechanism**—In addition to TG, other inhibitors such as DBHQ display a specificity for SERCA. Kinetic studies indicate that although inhibition by a variety of hydrophobic molecules is generally related to interference with global conformational changes of the enzyme (38), TG and DBHQ may actually bind to separate sites (39). We then tested whether the F256V mutation would interfere with the inhibitory effect of DBHQ. We found that DBHQ has an identical effect on the WT enzyme and on the F256V (Fig. 7), which is the most effective mutant in reducing the inhibitory effect of TG (Fig. 4 and Table I).

Another aspect of TG specificity is its lack of inhibitory effect on other cation transport ATPases (5). It is not known whether such specificity is because of lack of binding or an inefficiency of the bound drug. We then tested whether the ATPase activity of...
the drug transport P-glycoprotein may be influenced by TG. It is shown in Fig. 8 that a 4-fold activation of the P-glycoprotein ATPase is produced by TG with an apparent $K_m$ of 0.4 mM followed by reversal of this stimulation at higher concentrations ($K_i$ 13.5 mM) as is usually observed for other transport substrates in this enzyme (34). This indicates that TG binds with high affinity to membrane-bound transport ATPases of obvious structural diversity, but its ability to inhibit or stimulate catalytic activity is specifically dependent on the structure of the enzyme.

FIG. 6. Effect of TG on limited protease K digestion of WT ATPase, F834A, F256V, and I765G in the presence and in the absence of Ca$^{2+}$. Digestion with protease K for 40 min at 25 °C was performed as explained under "Experimental Procedures." The samples were digested in the presence (10 μM) or in the absence (1 mM EGTA) of Ca$^{2+}$. TG (1 μM) where indicated. The first lane in each panel is undigested WT or mutant ATPase. Note the protection (lanes 2 and 3) in the WT and (to a slightly lesser extent) in the F834A panels. No protection (lanes 2 and 3) is observed in the F256V and I765G panels.

FIG. 7. Sensitivity of WT ATPase and F256V mutant to DBHQ. Ca$^{2+}$ ATPase measurements and plots as for Fig. 4. ■, WT; ▲, F256V.

FIG. 8. Activation of P-glycoprotein ATPase by thapsigargin. WT human P-glycoprotein was expressed in yeast, purified, and reconstituted in liposomes. The ATPase activity was measured as described under "Experimental Procedures." The experimental points were fitted as described previously (34); $r^2 = 0.9115$. The basal activity rate was 0.55 ± 0.05 μmoles/mg protein/min, and the maximal stimulated rate was 2.50 ± 0.21 μmoles/mg protein/min. The stimulation $K_m$ was 0.38 ± 0.11 μM and the $K_i$ was 13.5 ± 2.8 μM.

DISCUSSION

Structural studies indicate that TG resides in a cavity delimited by the M3, M5, and M7 helices, near the cytosolic surface of the membrane (Figs. 1 and 9). Our mutational analysis shows that Phe-256 (M3), Ile-765 (M5), and Tyr-837 (M7) are most important for TG binding. It is apparent that the Phe-256 ring provides a complementary surface to TG, which is very important to the strength of binding. The effect produced
by the F256V mutation is consistent with analogous effects produced by corresponding changes on the TG structure (2). The Tyr-837 ring may be less important because of a misalignment in the native structure, whereby the Y837A alone is less disruptive. The role of Ile-765 is less clear, and the effect of its mutation is likely related to direct structural perturbation.

The effects of mutations on binding affinity are not dependent as much on removal of the native side chain as on the type and size of substitution. For instance, the mutation of Phe-256 to Val is more effective than to Leu, and the Ile-765 mutation to Gly is more effective than to Ala, evidently because of the length and hydrophobic character of the substitute chain. These findings are consistent with the idea that TG binding is determined by hydrophobic partitioning and complementary molecular shapes (38). Apparently, TG binding to the SERCA enzyme involves only one hydrogen bond with Ile-829 (15).

It should be pointed out that the ATPase activity of the Ile-765 mutant is somewhat low, i.e. ~40% of the WT (Table 1). We then considered whether the low apparent affinity of this mutant for TG may be a kinetic effect related to a slow step leading to formation of E2 and a consequent lower steady state level of this TG-sensitive intermediate. However, the samples were equilibrated with TG before starting the ATPase measurements, and the dissociation constant of TG from the inhibitory site is very slow (10^{-2}-10^{-3} s^{-1} as compared with 10 s^{-1} for the catalytic turnover). Consequently, the percentage of enzyme undergoing TG dissociation during an ATPase cycle is extremely low. Therefore, the observed inhibition is in practice related to the level of stable and inhibited enzyme-TG complex formed before starting the ATPase reaction. This argues in favor of a direct effect of the Ile-765 mutation on the TG binding site.

Interference by single mutations with the TG inhibitory effect, in addition to reduction of binding affinity, underlines the importance of structural specificity not only for TG binding but also for the inhibitory mechanism. This is more prominent in the F256V mutant but also significant in the I765A and Y837A mutants. Other mutations such as the F834A do not produce this effect possibly allowing some structural flexibility near the TG binding site. It is clear that TG would collide severely with
Phe-256 and Tyr-837 if the ATPase were to acquire the E1-Ca$^{2+}$ conformation (Fig. 9). Weaker collisions would also occur with Ile-765, whereas Gln-259 may be able to avoid collisions by changing its side chain conformation. In fact, the experiments on proteinase K digestion demonstrate that mutational interference with the inhibitory effect of TG on catalytic activity (Fig. 4 and Table I) and with Ca$^{2+}$-induced conformational change (Fig. 6) occur in parallel.

A most interesting aspect of our experiments is the demonstration that the inhibitory effect of TG involves a specific mechanism that is not shared by DBHQ, which is another SERCA inhibitor likely bound in a neighboring hydrophobic cleft. Furthermore, our experiments demonstrate that the specificity inhibitory effect of TG for SERCA, as opposed to other transport enzymes, is not related to the lack of binding but rather to the effect of the bound drug. In fact, the 4-fold stimulation of the P-glycoprotein ATPase activity observed in our experiments demonstrates clearly that the response to bound TG may be in the form of inhibition or stimulation, depending on the enzyme structure. It is known, in this regard, that the binding site of the P-glycoprotein undergoes a drug-induced conformational change resulting in ATPase activation coupled to drug transport (40, 41). It is very interesting that the structural consequences of high affinity binding of the same drug, result in total inhibition or strong stimulation of ATP utilization in two different transport enzymes.

Finally, our finding that TG stimulates the P-glycoprotein ATPase provides a better understanding of the resistance acquired by cell lines subjected to selective pressure with TG (19, 42). It was found that the resistant cells manifest up-regulation of SERCA expression with various Phe-256 mutations, but also up-regulation of the multidrug transport P-glycoprotein. This indicates that redundancy plays a role in the acquisition of resistance, including overexpression of the resistant SERCA mutant, as well as overexpression of the multidrug transporter P-glycoprotein. These findings may be helpful in the assessment of the possible use of TG in targeted cancer treatment (11).

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