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Molecular Biology and Mutagenesis—Human SERCA3a was sub-cloned into pcDNA3.1 using the Apal and AflII restriction sites. Primers were designed such that for each isoform, codon 256 was mutated to GTT, which encodes valine; substitution of phenylalanine to valine at amino acid position 256 was shown to confer the greatest resistance (about 40-fold) to thapsigargin for avian SERCA1 (14). The mutations were introduced using the QuikChange XL site-directed mutagenesis kit (Stratagene), following the manufacturer’s instructions.

Overexpression of Wild Type and F256V SERCA cDNA— COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium media supplemented with 10% fetal calf serum under 5% CO2, 95% air. COS-7 cells were cultured in 100-mm diameter Petri dishes to 80% confluence before being transiently transfected with wild type or F256V SERCA cDNA using TransFast (Promega). For each plate of cells, plasmid DNA (10 μg), TransFast (60 μl), and serum-free Dulbecco’s modified Eagle’s medium (5 ml) were added to a universal and vortexed for 30 s. The transfection media were left at room temperature for 15 min, after which it was vortexed as before. The media were removed from the COS-7 cells and replaced with the transfection media, and the cells were then cultured in transfection media for 3 h, after which 10 ml of Dulbecco’s modified Eagle’s medium prewarmed to 37 °C supplemented with 10% fetal calf serum was added to each Petri dish. The following day, the media were aspirated and replaced with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The cells were harvested after 48 h after transfection.

Preparation of COS-7 Microsomal Membranes—The method used is one modified from that described in Ref. 24. For the harvesting of five 10-cm diameter Petri dishes of cells, each 10-cm diameter dish of cells was washed with 5 ml of prewarmed (37 °C) phosphate-buffered saline solution. The cells were scraped in a solution of cold (prechilled on ice) phosphate-buffered saline with 5 mM EDTA before being transferred to a universal tube on ice. The cells were pelleted by centrifugation at 4000 × g for 15 min at 4 °C. The supernatant was discarded, and the resulting pellet was resuspended gently with 10 ml of phosphate-buffered saline prior to centrifugation, as before. The supernatant was then discarded, and the cells were resuspended gently with 5 ml of prechilled (on ice) hypotonic solution (10 mM Tris, pH 7.5, 0.5 mM MgCl2). The resuspended cells were then incubated on ice for 10 min prior to the addition of 0.1 mM phenylmethylsulfonyl fluoride and 4 μg/ml leupeptin. The lysed cells were homogenized using a Polytron for 10 s and further homogenized with a Potter homogenizer for 30 s. The homogenate was then diluted with an equal volume of buffer (0.5 M sucrose, 6 mM 2-mercaptoethanol, 40 μM CaCl2, 300 mM KCl, and 20 mM Tris, pH 7.5) before being centrifuged at 10000 × g for 10 min at 4 °C. The supernatant was removed and made up to 0.6 M with KCl by the addition of an appropriate volume of a 2.5 M solution, prior to centrifugation at 100,000 × g for 60 min at 4 °C to obtain the microsomal membrane fraction. The microsomal pellet was then resuspended in buffer (0.25 M sucrose, 0.15 M KCl, 3 mM 2-mercaptoethanol, 20 μM CaCl2, and 10 mM Tris, pH 7.5). The microsomal membranes were rehomogenized by hand using a Potter homogenizer before being aliquoted and snap-frozen with liquid nitrogen, prior to storage at −80 °C. Analysis of SERCA Overexpression by Immunoblotting—Microsomal membranes prepared from non-transfected and transiently transfected COS-7 cells were resolved on 7.5% SDS-PAGE gels (25). Resolved protein samples were transferred onto nitrocellulose for 1 h at 750 mA in transfer buffer (0.1% w/v SDS, 20% v/v methanol, 25 mM Tris, and 190 mM glycine) (26).

Non-transfected and SERCA1b transiently overexpressing COS-7 microsomal membrane proteins were probed with the SERCA1 monoclonal antibody 1/2H7 ascites fluid diluted at a concentration of 1 in 20 for 1.5 h and with secondary horseradish peroxidase-conjugated antigoat IgG (Sigma). Non-transfected and SERCA2b transiently overexpressing microsomal membrane proteins were probed with SERCA2 N19 antibody (Santa Cruz Biotechnology, sc-8093) diluted 1 in 100 for 1.5 h and with secondary horseradish peroxidase-conjugated anti-goat IgG (Sigma). Non-transfected and SERCA3a transiently overexpressing microsomal membrane proteins were probed with the anti-SERCA3 monoclonal antibody PL/IM430 (Chemicon) diluted to a concentration of 1 μg/ml and with secondary horseradish peroxidase-conjugated antigoat IgG. Following incubation with secondary antibodies, the blots were incubated with SuperSignal West Pico enhanced chemiluminescent substrate (Pierce) before being used to expose Kodak BioMax MR film, which was developed using an XOgraph.

Measurement of the Ca2+-dependent ATPase Activity of SERCA-Overexpressing Microsomal Membranes—The Ca2+-dependent ATPase activity of COS-7 cell microsomal membranes was measured using the phosphate liberation assay, as described by Longland et al. (27). Briefly, microsomal extracts (typically 5 μg) were resuspended in 200 μl of buffer (45 mM Hepes/KOH (pH 7.0), 6 mM MgCl2, 2 mM NaN3, 0.25 mM sucrose), supplemented with 5 μg/ml A23187 ionophore and EGTA and CaCl2 to give a free [Ca2+] of 2 μM, which was determined to be optimal for all isoforms. Assays were preincubated at 37 °C for 10 min prior to the addition of ATP with a final concentration of 6 mM to initiate activity. The reactions were then incubated at 37 °C for 40 min, which was shown to be linear, before the addition of 50 μl of 6.5% trichloroacetic acid, and the reactions were then stored on ice for 10 min before centrifugation for 5 min at 20000 × g. Supernatant (100 μl) was added to 1.50 μl of buffer (11.25% v/v acetic acid, 0.25% w/v copper sulfate, and 0.2 mM sodium acetate, pH 4.0). Ammonium molybdate solution (5% w/v, 25 μl) was then added, followed by the addition of 25 μl of ELAN reagent (2% w/v p-methyl-aaminophenol sulfate and 5% w/v sodium sulfite). The samples were mixed, and the blue coloration was allowed to develop for 10 min prior to measuring the absorption at 870 nm using a Dynatech Laboratories enzyme-linked immunosorbent assay plate reader. The amount of P2 liberated was determined by comparison with known phosphate standards. The activities were also determined in the absence of the addition of Ca2+ to determine non-
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TABLE 1

Ca\(^{2+}\)-dependent ATPase activity of COS-7 cell membranes overexpressing SERCA WT and mutant (F256V) isoforms

Non-transfected COS-7 cell membranes had a Ca\(^{2+}\)-dependent ATPase activity of 4 ± 1 pmol/min/mg. The activities represent the mean ± S.E. of between 3–6 replicates.

| Preparation     | SERCA1b WT | SERCA1b F256V | SERCA2b WT | SERCA2b F256V | SERCA3a WT | SERCA3a F256V |
|-----------------|------------|---------------|------------|---------------|------------|---------------|
| pmol/min/mg     |            |               |            |               |            |               |
| 1               | 49 ± 3     | 111 ± 2       | 92 ± 1     | 26 ± 1        | 27 ± 2     | 28 ± 2        |
| 2               | 28 ± 2     | 29 ± 2        | 25 ± 2     | 37 ± 2        | 56 ± 2     | 56 ± 2        |

Fig. 2. Inhibition of SERCA isoforms and their corresponding F256V mutants by thapsigargin. The activities of the COS-7 cell membranes overexpressing the SERCA proteins were measured at 37 °C and pH 7.2 as described under “Materials and Methods.” An example of the inhibition of SERCA1b having an apparent inhibition constant (\(K_i\)) of 0.21 ± 0.05 nM, whereas the potency for thapsigargin for the F256V mutant of SERCA1b as expected was much reduced with a \(K_i\) of 45 ± 22 nM, indicating a shift in potency of inhibition in the order of about 200-fold. A comparison of the potency of thapsigargin for the wild type isoforms of SERCA2b and SERCA3a (Fig. 2, B and C) showed that there was a difference in their sensitivities (the \(K_i\) for wild type SERCA2b and SERCA3a were determined to be 1.3 ± 0.5 and 12 ± 6 nM, respectively). This therefore shows that although thapsigargin is a potent inhibitor of all SERCA isoforms, there is a measurable difference in the potency between isoforms with SERCA1b being the most sensitive and SERCA3a the least by a factor of almost 60-fold. In addition, these differences were unlikely to be linked to the degree of SERCA expression since there was no correlation in TG potency between high and activity preparations. Comparing the effects of the F256V mutation in SERCA2b and SERCA3a with the corresponding wild type isoforms also showed that this mutation decreased the thapsigargin sensitivity in the other isoforms. For SERCA2b, the \(K_i\) shifted from 1.3 ± 0.5 nM for the wild type to 6.3 ± 3.0 nM (indicating a

RESULTS

COS-7 cells were transfected with wild type and F256V SERCA plasmid DNA, and microsomal membranes were prepared as described under “Materials and Methods.” The overexpression of each isoform of wild type and F256V SERCA was analyzed by immunoblotting with isoform-specific anti-SERCA antibodies. Fig. 1 illustrates typical immunoblots using isoform-specific antibodies to show that the COS-7 cells were successfully transfected to express SERCA1b, SERCA2b, and SERCA3a isoforms of the Ca\(^{2+}\) ATPase. Mutant F256V of all three isoforms were also expressed.

Table 1 shows the Ca\(^{2+}\)-dependent ATPase activity as assessed by the hydrolysis of ATP from membranes extracted from the transfected COS-7 cells. The activities were measured at a free Ca\(^{2+}\) concentration of 2 \(\mu\)M, which we found to be optimal for all isoforms and mutants. During the course of these studies, a number of COS-7 cell membrane preparations were produced for both wild type and F256V mutants of the SERCA isoforms. As can be seen, the activities were variable between preparations even of the same isoform, which we have attributed to variations in transfection efficiency (which was determined to be typically between 30 and 60%) and protein degradation/denaturation during membrane preparation.

Fig. 2, A–C, shows the effects of thapsigargin on Ca\(^{2+}\)-ATPase activity between the three isoforms and their corresponding F256V mutations. Fig. 2A shows that thapsigargin is an extremely potent inhibitor of SERCA1b having an apparent inhibition constant (\(K_i\)) of 0.21 ± 0.05 nM, whereas the potency for thapsigargin for the F256V mutant of SERCA1b as expected was much reduced with a \(K_i\) of 45 ± 22 nM, indicating a shift in potency of inhibition in the order of about 200-fold. A comparison of the potency of thapsigargin for the wild type isoforms of SERCA2b and SERCA3a (Fig. 2, B and C) showed that there was a difference in their sensitivities (the \(K_i\) for wild type SERCA2b and SERCA3a were determined to be 1.3 ± 0.5 and 12 ± 6 nM, respectively). This therefore shows that although thapsigargin is a potent inhibitor of all SERCA isoforms, there is a measurable difference in the potency between isoforms with SERCA1b being the most sensitive and SERCA3a the least by a factor of almost 60-fold. In addition, these differences were unlikely to be linked to the degree of SERCA expression since there was no correlation in TG potency between high and activity preparations. Comparing the effects of the F256V mutation in SERCA2b and SERCA3a with the corresponding wild type isoforms also showed that this mutation decreased the thapsigargin sensitivity in the other isoforms. For SERCA2b, the \(K_i\) shifted from 1.3 ± 0.5 nM for the wild type to 6.3 ± 3.0 nM (indicating a...
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Table 2 lists the inhibition constants for some inhibitors for the different wild type isoforms and corresponding F256V mutants. Table 2 shows that for SERCA2b, mutation of Phe-256 to Val has only at best a decrease in potency of about 5-fold. For SERCA3a, the $K_i$ values for a variety of other commonly used Ca$^{2+}$ ATPase and well characterized inhibitors on both the wild type and the F256V mutant SERCA isoforms 1b, 2b, and 3a. For SERCA1b, the $K_i$ for CPA is increased from 90 nM for SERCA1b to 2.5 μM and 600 nM for SERCA2b and SERCA3a, respectively. Thus there also appears to be a substantial difference in the sensitivity of the F256V mutation.

TABLE 2

| Inhibitor | Rabbit SERCA1b (WT) $K_i$ | Rabbit SERCA1b (F256V) $K_i$ | Human SERCA2b (WT) $K_i$ | Human SERCA2b (F256V) $K_i$ | Human SERCA3a (WT) $K_i$ | Human SERCA3a (F256V) $K_i$ |
|-----------|-------------------------|-----------------------------|--------------------------|-----------------------------|--------------------------|-----------------------------|
| TG        | $2.1 \times 10^{-10}$   | $4.5 \times 10^{-8}$        | $1.3 \times 10^{-9}$     | $6.3 \times 10^{-9}$        | $1.2 \times 10^{-8}$     | $1.3 \times 10^{-6}$       |
| CPA       | $9.0 \times 10^{-8}$    | $1.9 \times 10^{-7}$        | $2.5 \times 10^{-6}$     | $1.0 \times 10^{-6}$        | $6.0 \times 10^{-7}$     | $7.0 \times 10^{-7}$       |
| BHQ       | $7.0 \times 10^{-6}$    | $2.1 \times 10^{-5}$        | $2.6 \times 10^{-4}$     | $6.7 \times 10^{-4}$        | $1.7 \times 10^{-3}$     | $2.7 \times 10^{-3}$       |
| NP        | $2.38 \times 10^{-5}$   | $1.45 \times 10^{-5}$       | $3.5 \times 10^{-4}$     | $2.1 \times 10^{-4}$        | $4.3 \times 10^{-4}$     | $1.86 \times 10^{-5}$      |

The numbers in brackets for the WT SERCA2b and SERCA3a indicate the fold change in inhibition constants for the inhibitors compared to SERCA1b (WT).

The numbers in brackets for the mutant (F256V) SERCA1b, SERCA2b, and SERCA3a indicate the fold change in inhibition constants for the inhibitors compared to the corresponding wild types.

A comprehensive study was undertaken to assess the effects of other inhibitors on the different isoforms of SERCA and the decrease in potency of only about 5-fold. For SERCA3a, the $K_i$ shifted from 12 ± 6 nM for the wild type to 1.3 ± 0.4 μM for F256V, indicating a decrease in potency of more than a 100-fold. Therefore it would appear that for SERCA2b, mutation of Phe-256 to Val has only at best a rather modest effect.

![FIGURE 3. Effects of 2-APB on the activity of the SERCA isoforms and their corresponding F256V mutants.](image)

![FIGURE 4. Effects of curcumin on the activity of the SERCA isoforms and their corresponding F256V mutants.](image)
Effects of Inhibitors on SERCA Isoforms

FIGURE 5. The thapsigargin binding site of SERCA. A, shown are 5 amino acid residues (Phe-256, Ile-765, Ile-829, Phe-834, and Tyr-837) that form part of the thapsigargin binding site of SERCA1 (Protein Data Bank code 1IWO). B shows the predicted structure of the thapsigargin binding site in SERCA1 when Phe-256 is mutated to Val-256. (The structure in black is thapsigargin). C shows the model of the location and the orientation of these residues predicted by SWISS-MODEL for SERCA2 (white) superimposed onto SERCA1 (gray) and aligned with reference to Phe-256. (Note that the corresponding residues Ile-764, Ile-828, Phe-833, and Tyr-836 are displaced by one amino acid when compared with SERCA1 for accurate alignment). D shows the same type of superimposition of SERCA3 (white) and SERCA1 (gray).

sitivity of CPA between the isoforms, with SERCA2b being especially insensitive to CPA when compared with the other SERCA isoforms. This difference, however, is not so apparent when comparing the $K_i$ values for CPA with the F256V mutants for all three isoforms. When comparing the $K_i$ values between wild type and F256V mutants for CPA, the changes observed are at best only 2–3-fold, which would indicate that this mutation has little effect upon CPA binding. This is in agreement with another study comparing the effects of CPA on the avian SERCA1 and the corresponding F256V mutant (28). When comparing the $K_i$ values for the three isoforms caused by BHQ inhibition, there appears to be very little difference between them. In addition, there was little substantial difference in inhibition between the wild type isoforms and their corresponding F256V mutants. Thus it must be concluded that BHQ binding is likely to be in a region of great similarity between the three isoforms but distinct from the region around Phe-256 encompassing the thapsigargin binding site, a conclusion drawn by others using inhibitor competition studies (29).

Both the wild type forms of SERCA2b and SERCA3a appear to be more sensitive than SERCA1b for inhibition by NP. However, these differences are modest as the $K_i$ values show relatively small differences. The $K_i$ values for nonylphenol between the three isoforms range from about 3.5 $\mu M$ for SERCA2b to 23.8 $\mu M$ for SERCA1b. Therefore unlike thapsigargin and CPA, where SERCA1b is the most sensitive isoform, in the case of NP, it appears that SERCA1b is the least sensitive. Again the $K_i$ values are relatively little affected for the F256V mutation when compared with the corresponding wild type isoforms, except for SERCA3a, where there was a 4-fold shift to lower sensitivity.

Fig. 3, A and B, shows the effects of 2-APB on the activity of the three SERCA isoforms (Fig. 3A) and their corresponding F256V mutants (Fig. 3B). The potency of inhibition by this inhibitor is considerably less than for the other inhibitors tested with the $K_i$ values in the mM range; however, previous mutagenesis and modeling studies have suggested that 2-APB binds in a hydrophobic pocket near to or possibly within the site to which thapsigargin binds (21). The effects of 2-APB on the wild type activity of both SERCA1b and SERCA3a were almost identical ($K_i$ values 0.46 ± 0.10 and 0.44 ± 0.06 $\mu M$, respectively). The $K_i$ value for SERCA2b was substantially different at 1.5 ± 0.2 $\mu M$. For the F256V mutants, the $K_i$ values for SERCA2b and SERCA3a were similar to those for the corresponding wild types (1.9 ± 0.4 and 0.22 ± 0.1 $\mu M$ for SERCA2b and SERCA3a, respectively). However, there was a modest difference for the F256V mutant SERCA1b when compared with the wild type (i.e. the $K_i$ was 1.4 ± 0.2 $\mu M$, which is about 3-fold higher than wild type form).

The SERCA inhibition by curcumin (Fig. 4) also showed differences in potency between the isoforms, with SERCA1b and SERCA3a having similar $K_i$ values (5.8 ± 1.6 and 8.6 ± 2.5 $\mu M$, respectively). The inhibition of SERCA2b by curcumin was substantially less potent with a $K_i$ of 53 ± 6 $\mu M$. Although both of the $K_i$ values for the F256V mutants of SERCA1b and SERCA3a were shifted to lower sensitivity by about 3-fold ($K_i$ values 21 ± 3 and 25 ± 7 $\mu M$, respectively), it was, however, SERCA2b that showed the largest difference. The F256V mutant of SERCA2b showed a dramatic stimulation in activity at low concentrations of curcumin (up to 20 $\mu M$), where there was up to a 40% increase in activity. At higher concentrations, inhibition was again observed ($K_i$ was 60 $\mu M$ ± 10).

DISCUSSION

Our experiments clearly demonstrated expression of SERCA1, SERCA2, and SERCA3 in COS-7 cells as assessed by Western blotting using isoform-specific antibodies and that this expression was considerably higher than endogenous SERCA levels in COS-7 cells from activity studies (non-transfected COS-7 cell membranes had a Ca$^{2+}$-ATPase activity of typically 4 pmol/min/mg when compared with up to more than 100 pmol/min/mg in optimally transfected cells). From Table 1, it...
can be seen that the degree of activity of the transfected COS-7 cell membranes was variable between preparations even of the same isoform; however, even the lowest value for the transfected cell membranes was about 6-fold higher than endogenous untransfected levels. Therefore even in this preparation, over 80% of the Ca\(^{2+}\)-ATPase activity measured would come from the expressed SERCA protein. Although we tried, as far as possible, to transfect the cells using identical procedures, this variability was likely to be due to a number of factors that are difficult to control. These included the condition of cells prior to transfection, the number of passages of the cells prior to transfection, and also variability in membrane extraction (which we have previously shown to be a major factor in the variability of Ca\(^{2+}\)-ATPase activity between sarcoplasmic reticulum preparations (30)).

The results presented in Fig. 2 show that SERCA isoforms vary in their sensitivity to thapsigargin by a factor of about 60-fold when comparing SERCA1b with SERCA3a. However, since the \(K_i\) values varied from \(~0.2\) nM for SERCA1b to 12 nM for SERCA3a, thapsigargin must still be considered a very potent inhibitor of all SERCA isoforms. The \(K_i\) for SERCA1 of 0.2 nM is similar to that found in other studies in which SERCA1 was overexpressed (11). The inhibition by thapsigargin of SERCA2b was found to have an apparent \(K_i\) of \(~1\) nM and was completely inhibited by 30 nM thapsigargin, in agreement with the value of 25 nM observed for SERCA2b-expressing COS-1 membranes (11). The finding that SERCA3a was found to be the least sensitive to thapsigargin was surprising since thapsigargin has been shown to stabilize the E2 form of the ATPase (9) and SERCA3 is also believed to favor the E2 form when compared with SERCA1 and SERCA2 (31). Therefore it is likely that SERCA3 has a slightly less than optimal thapsigargin binding domain than SERCA1. As a high resolution x-ray crystal structure of thapsigargin bound to SERCA1 has been solved, the amino acid residues lining the thapsigargin binding domain have been identified (15). From structural analysis and mutagenesis studies, several amino acid residues that are important for thapsigargin binding and inhibition have been identified and include: Phe-256 (on TM3) Ile-765 (on TM5), Ile-828 (on TM6), and Ile-829 (Fig. 5) (15, 33). This hindrance, however, would also be seen in the models for F256V mutants of SERCA2 and therefore cannot account for the small effect that this mutation has on TG inhibition in SERCA2. However, it cannot be excluded that there are different conformations of the amino acids within the thapsigargin binding site that are not predicted from these models.

When compared with the effects of thapsigargin, both on the different SERCA isoforms and on the F256V SERCA mutants, the other inhibitors investigated in this study generally showed rather modest differences. When assessing the effects of these inhibitors on the different SERCA isoforms, only CPA showed a considerable difference in its potency between the three isoforms (i.e. \(~30\)-fold between wild type SERCA1 and SERCA2 and 4-fold between SERCA2 and SERCA3). However, when comparing the effects of CPA between the wild type and F256V mutants of the isoforms, only at best a 2–3-fold change was observed. The other commonly employed inhibitor, BHQ, showed little difference between the isoforms and little difference in potency for all three F256V mutants. This latter finding is in agreement with that of Ref. 29, and therefore, one must conclude that both CPA and BHQ bind to different sites to thapsigargin. For nonylphenol, both SERCA2b and SERCA3a showed a significant increase in sensitivity when compared with SERCA1b. The corresponding F256V mutants of all the isoforms were, however, little different from their corresponding wild types. SERCA2b showed a much reduced effect from curcumin than the other isoforms, and all showed a modest but significant reduction in potency upon introduction of the F256V mutation, which would argue that curcumin is likely to be binding in the near vicinity of the thapsigargin binding site.

In a previous study of 2-APB on the Ca\(^{2+}\)-ATPase (21), from mutational and modeling studies, we indicated that 2-APB could bind at one of two sites: one being close to the L6–7 loop and the other being within the thapsigargin site. Since the F256V mutation had no effect on the potency of SERCA2b and SERCA3a and only a modest effect for SERCA1b, this would indicate that 2-APB is more likely to bind at a site close to the L6–7 loop.

From the data presented here, it is unlikely that many of the commonly used inhibitors are likely to bind within the known thapsigargin binding site. In addition, several of the inhibitors caused their effects by stabilizing the ATPase in an E2 form like thapsigargin (i.e. BHQ and NP). Therefore these inhibitors must be binding to other hydrophobic
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binding sites on SERCA that cause a similar or “global”-induced conformational change to inhibit the ATPase by similar mechanisms (29).

In summary, this study has demonstrated that the sensitivity to thapsigargin is different for the SERCA isoforms and that the reduction in thapsigargin sensitivity caused by the F256V mutation is also different for the three isoforms, with SERCA2b only being modestly affected by this mutation. In addition, although some of the other inhibitors investigated do show some differences in their sensitivity toward the different SERCA isoforms, most are little affected by the F256V mutation, indicating that they inhibit the Ca\(^{2+}\)-ATPase by binding to hydrophobic sites distinct to that where thapsigargin binds.

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