The Golgi/secretory pathway Ca\(^{2+}\)/Mn\(^{2+}\)-transport ATPase (SPCA1a) is implicated in breast cancer and Hailey-Hailey disease. Here, we purified recombinant human SPCA1a from *S. cerevisiae* and measured Ca\(^{2+}\)-dependent ATPase activity following reconstitution in proteoliposomes. The purified SPCA1a displays a higher apparent Ca\(^{2+}\) affinity and a lower maximal turnover rate than the purified sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA1a). The lipids cholesteryl hemisuccinate, linoleamide/oleamide, and phosphatidyethanolamine inhibit and phosphatidic acid and sphingomyelin enhance SPCA1a activity. Moreover, SPCA1a is blocked by micromolar concentrations of the commonly used SERCA1a inhibitors thapsigargin (Tg), cyclopiazonic acid, and 2,5-di-tert-butylhydroquinone. Because tissue-specific targeting of SERCA2b by Tg analogues is considered for prostate cancer therapy, the inhibition of SPCA1a by Tg might represent an off-target risk. We assessed the structure-activity relationship (SAR) of Tg for SPCA1a by *in silico* modeling, site-directed mutagenesis, and measuring the potency of a series of Tg analogues. These indicate that Tg and the analogues are bound via the Tg scaffold but with lower affinity to the same homologous cavity as on the membrane surface of SERCA1a. The lower Tg affinity may depend on a more flexible binding cavity in SPCA1a, with low contributions of the Tg O-3, O-8, and O-10 chains to the binding energy. Conversely, the protein interaction of the Tg O-2 side chain with SPCA1a appears comparable with that of SERCA1a. These differences define a SAR of Tg for SPCA1a distinct from that of SERCA1a, indicating that Tg analogues with a higher specificity for SPCA1a can probably be developed.

As an important secondary messenger, the partitioning of intracellular Ca\(^{2+}\) controls a wide range of cellular responses, including contraction, secretion, cell growth, differentiation, proliferation, and cell death (1, 2). A sub-micromolar basal cytosolic Ca\(^{2+}\) concentration range is strictly maintained by intracellular Ca\(^{2+}\) pumps, whereas Ca\(^{2+}\) channel activity produces confined spatial and temporal cytosolic Ca\(^{2+}\) signals up to 10–100 μM that control various cellular activities. Ca\(^{2+}\) transporters belonging to the P2-type Ca\(^{2+}\) transport ATPases maintain the low housekeeping cytosolic Ca\(^{2+}\) levels and reduce the cytosolic Ca\(^{2+}\) concentration when a Ca\(^{2+}\)-signaling event ceases. The P2-type ATPases encompass the plasma membrane Ca\(^{2+}\)-ATPase, SERCA, and SPCA Ca\(^{2+}\) pumps that perform active Ca\(^{2+}\) transport across the plasma membrane, the intracellular membranes of the sarco(endo)plasmic reticulum, and the Golgi/secretory pathway, respectively (3). SERCA and SPCA belong to the subfamily P2A and share around 30% sequence identity and over 40% similarity (4).

In humans, the SPCA proteins are encoded by two genes (*ATP2C1* and *ATP2C2*) (4–7). SPCA1 is the housekeeping isoform, which by transporting Ca\(^{2+}\) and Mn\(^{2+}\) into the Golgi lumen supports essential Golgi/secretory pathway functions. Ca\(^{2+}\)/Mn\(^{2+}\) transport is required for proper protein folding, enzymatic activities (e.g. glycosylation), sorting and/or trafficking of proteins (8). In conjunction with SERCA, this ATPase also provides Ca\(^{2+}\)-storage pools for subsequent Ca\(^{2+}\)-release events (4, 9, 10). A total of four SPCA1 splice variants exist, SPCA1a–d, differing only at the C terminus. Although SPCA1a is considered to be the housekeeping variant, the differential tissue/subcellular distributions of the SPCA1 isoforms remain.

---

**References**

1. To whom correspondence should be addressed: Laboratory of Cellular Transport Systems, Dept. of Cellular and Molecular Medicine, ON1 Campus Gathuisberg, KU Leuven, Herestraat 49/Box 802, B3000 Leuven, Belgium. Tel.: 32-16-330720; E-mail: peter.vangheluwe@kuleuven.be.

2. The abbreviations used are: SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\) -ATPase; Tg, thapsigargin; PSA, prostate-specific antigen; SAR, structure-activity relationship; CPA, cyclopiazonic acid; BHQ, 2,5-di-tert-butylhydroquinone; BP (bisphenol), 2,2’-methylenebis(6-tert-butyl-4-methylphenol); ANOVA, analysis of variance; MOE, molecular operating environment; PDB, Protein Data Bank; BisTris, 2-[bis-(hydroxymethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; 2-ME, 2-mercaptoethanol; DDM, dodecyl β-D-maltoside; Ni-NTA, nickel-nitritriacetic acid; PC, phosphatidylcholine; CL, cholesterol; SM, sphingomyelin; PA, phosphatidic acid; PE, phosphatidylethanolamine; CHEMS, cholesteryl hemisuccinate; CV, column volume; PS, phosphatidylserine; SPCA, Golgi/secretory pathway Ca\(^{2+}\)-ATPase.
largely unexplored (11). The more restricted expression pattern of SPCA2 in cultured hippocampal neurons, colon, secretory acini, and luminal epithelial cells of mouse mammary tissue suggests that the second isoform may play a more specialized function, for instance in secretion (6, 12–14).

In humans, SPCA1 haploinsufficiency, due to heterozygous loss-of-function mutations, causes Hailey-Hailey disease (OMIM169600), a heritable autosomal-dominant skin disorder characterized by recurrent skin vesicles and erosions that typically arise in adulthood (15, 16). In addition, SPCA1a and/or SPCA2 proteins are up-regulated in mammary gland during lactation and in various breast cancer sub-types thereby contributing to a pathological Ca$^{2+}$ dyshomeostasis (17, 18). Independent of its transport activity, SPCA2 interacts with and activates the plasma membrane Ca$^{2+}$ channel Orai1 via the N and C termini (18), leading to store-independent Ca$^{2+}$ entry (18) and subsequent Ca$^{2+}$ transfer into the secretory pathway (19). During lactation, this system contributes to the cellular uptake of Ca$^{2+}$ in mammary gland epithelial cells for the subsequent release of Ca$^{2+}$ into the milk (20).

In general, SPCA1 isoforms display a higher Ca$^{2+}$ affinity but a lower maximal turnover rate than SERCA isoforms (21), but so far, a detailed functional analysis of SPCA isoforms has remained difficult due to the confounding presence of other abundant housekeeping Ca$^{2+}$-ATPases in cellular membrane fractions. In addition, although several potent SERCA inhibitors (thapsigargin (Tg), cyclopiazonic acid (CPA), and 2,5-di-tetrahydroxybutylhydroquinone (BHQ)) are widely used to selectively inhibit endoplasmic reticulum Ca$^{2+}$ transport (4, 22), selective pharmacological tools for SPCA inhibition are lacking. Because of the close sequence homology between SERCA and SPCA isoforms, several of the so-called highly selective SERCA inhibitors are also reported to inhibit SPCA in isolated membrane fractions of various organisms, albeit at much higher concentrations (4, 23–25).

The inhibition of SPCA1a by Tg may give rise to some concerns in connection with the clinical use of Tg analogues that target SERCA for prostate cancer therapy. In this therapeutic approach, a non-toxic, cell-impermeable pro-drug is administered that consists of a Tg analogue, which is coupled to a peptide that is specifically cleaved by the prostate-specific antigen (PSA) protease (26, 27). Extracellular cleavage of the peptide by local PSA in or near the prostate cancer releases the toxic Tg analogue Leu-8ADT, which is taken up by neighboring PSA-excreting cancer cells inducing targeted cell death (27). The estimated 3 orders of magnitude difference in the binding affinity of Tg for SERCA and SPCA may be considered sufficiently discriminatory to enable the use of Tg analogues for selective SERCA inhibition in vitro and in vivo. However, it remains unclear whether this is also true for other Tg analogues.

Toward the design of Tg analogues for clinical therapy related to SPCA dysfunction (see “Discussion”), we wanted to understand the off-target effect of Tg on SPCA1a, the closest relative of SERCA, by determining the structure-activation relationship (SAR) of Tg for SPCA1a. Moreover, due to the reported micromolar affinity of Tg to SPCA1 (24), one might consider Tg as a possible lead compound for the development of an SPCA1a inhibitor. Insights in the SAR of Tg for SPCA1a may aid the design of specific SPCA1a inhibitors as powerful pharmacological tools.

In this study, we purified the human SPCA1a isoform from a yeast recombinant expression system and reconstituted it into lipid vesicles. This purified system eliminates the influence of other membrane-bound contaminants or Ca$^{2+}$-ATPases, allowing the biochemical characterization of SPCA1a relative to purified and reconstituted SERCA1a. In particular, we demonstrate that SPCA1a activity is sensitive to the lipid environment, and we show that Tg and cholesteric compound sensitivity of SPCA1a may depend on the same or the overlapping binding pocket formed by transmembrane helices M3, M5, and M7. Finally, we have established the SAR of Tg for SPCA1a to facilitate the design of more selective SPCA inhibitors.

**Results**

**Affinity purification of recombinant human SPCA1a from Saccharomyces cerevisiae**

The relatively low expression level of SPCA1a in native tissues, typically in a background of high SERCA expression, hampers biochemical analysis of SPCA1a. As an alternative approach, we purified His$_7$-tagged human SPCA1a via Ni-NTA affinity chromatography from S. cerevisiae, which serves as an excellent model system to produce and purify P-type transport ATPases (8). SDS-PAGE analysis reveals that SPCA1a monomer and dimer bands were highly enriched in the elution fractions (Fig. 1A). Using mass spectrometry, we observed a molecular mass of 101.8 kDa, close to the predicted molecular mass of 101.7 kDa and high purity (>95%) of the human SPCA1a (Fig. 1B). The concentrated His-tagged SPCA1a in the elution fraction was further demonstrated by immunoblotting with SPCA1 (Fig. 1C) and His tag (Fig. 1D)-specific antibodies.
Inhibition of SPCA1a by thapsigargin

SPCA1a reconstitution into proteoliposomes restores Ca\(^{2+}\)-dependent ATPase activity

We found that purified and lipid-free SPCA1a became reversibly inactivated in detergent solution (Fig. 2E). This we considered to be a result of either a removal of specific lipid component(s) essential for activity or loss of physical membrane constraints originally imposed by the membrane lipids. To restore a functional lipid environment, we reconstituted as described under “Experimental procedures” the proteoliposomes from detergent-solubilized SPCA1a by addition of defined lipid components, followed by removal of detergent with Bio-beads. For this, 600 µg of phosphatidylcholine (PC) was first mixed with 200 µg of purified and detergent-solubilized SPCA1a (i.e. the protein was solubilized with detergent at a lipid/protein ratio of 3:1, w/w). The efficiency of SPCA1a incorporation into liposomes was compared for three types of detergents, i.e. Triton X-100, DDM, or C12E8 (28, 29). Reconstitution with Triton X-100 rendered the most efficient incorporation of SPCA1a into proteoliposomes, especially at a high lipid to detergent ratio (Fig. 2A). Importantly, we were able to recover the Ca\(^{2+}\)-dependent ATPase activity of the purified SPCA1a reconstituted with Triton X-100 or DDM but not with C12E8. Moreover, we determined that a lipid/detergent ratio of 20:1 generated the most active SPCA1a in proteoliposomes (Fig. 2A).
Inhibition of SPCA1α by thapsigargin

Lipids phosphatidic acid, sphingomyelin, cholesterol, and linoleamide/oleamide modulate SPCA1α activity

Membrane proteins are functionally adapted to the local lipid environment of their cellular membranes. We therefore analyzed the impact of different lipid types on the Ca\(^{2+}\)-dependent ATPase activity of SPCA1α, which in human colon adenocarcinoma cells is sensitive to cholesterol (CL) and is preferentially associated with lipid rafts (30). For reconstitution, various phospholipids (Fig. 2C), CL, sphingomyelin (SM), or a combination of the latter two (SM + CL) (Fig. 2D) were supplied together with PC, and SPCA1α ATPase activity was assessed. Compared with PC alone, the presence of phosphatidic acid (PA), SM, or SM/CL slightly but significantly enhanced activity, whereas phosphatidylethanolamine (PE) lowered the SPCA1α ATPase activity. In further experiments, PA was routinely added during SPCA1α reconstitution, as has been done previously for SERCA1α reconstitution (29), although we cannot exclude that PA may be a low-abundance lipid in the native Golgi membrane (31, 32).

Next, we directly compared the biochemical properties of purified and reconstituted SPCA1α (4:1 PC/PA ratio) and SERCA1α, the skeletal muscle Ca\(^{2+}\)-transport ATPase, which was purified and reconstituted according to a well-established protocol (29) (with a 9:1 PC/PA ratio as in Ref. 33) (Fig. 2E). Compared with SERCA1α, SPCA1α displays a higher apparent Ca\(^{2+}\) affinity (K\(_{m}\) of 0.125 ± 0.004 and 0.088 ± 0.005 \(\mu\)M, respectively) and a 5-fold lower turnover rate (V\(_{max}\) of 12.29 ± 0.13 and 2.54 ± 0.04 nmol of P\(_i\)/\(\mu\)g/min), in line with previous reports (21).

Although we previously reported that SPCA1α and SERCA1α respond differentially to CL (30), we observed here little functional effects of incorporating 20% CL upon reconstitution. However, by replacing CL with cholesteryl hemisuccinate (CHEMS), a more soluble cholesterol derivative (Fig. 2F), we found that it affected both the reconstituted SPCA1α or SERCA1α activities (Fig. 2G). SERCA1α displayed a concentration-dependent inhibition by CHEMS with an IC\(_{50}\) (defined as the concentration that leads to 50% inhibition of activity) of 8.8 ± 0.66 \(\mu\)M and a maximal inhibition of 80% at around 100 \(\mu\)M. For SPCA1α, a slight and not significant activation up to 106% was observed at lower concentrations, whereas higher concentrations above 6 \(\mu\)M impaired SPCA1α to a maximal inhibition of 47% at 300 \(\mu\)M, in line with the previous observation that SPCA1α and SERCA1α respond differentially to CL (30).

Because two endogenous lipids oleamide and linoleamide have recently been reported to be inhibitors of SPCA2 (34), we tested here whether these lipids also modify SPCA1α or SERCA1α activity (Fig. 3, A and B). Up to 1 mm, oleamide only partially inhibited SPCA1α and SERCA1α (Fig. 3A), whereas complete inhibition was previously reported for SPCA2 (34). In contrast to SERCA1α, linoleamide fully inhibited SPCA1α with an IC\(_{50}\) of 30 \(\mu\)M (Fig. 3B), which is 8-fold higher than the value that has been reported for SPCA2 (34), although a side by side SPCA1α/2 comparison could not be performed.

Figure 3. Dose-response effects of SPCA1α and SERCA1α inhibitors.
Dose-response curves of oleamide (A) and linoleamide (B) on SPCA1α and SERCA1α. Dose-response curves for the inhibition of SPCA1α (C) and SPCA1α (D) by Tg, CPA, BHQ, and BP. n = 3 for all experiments.

Several SERCA inhibitors block SPCA1α activity at micromolar concentration

To further validate the reconstituted SPCA1α system, we verified whether bisphenol (35, 36) and the SERCA inhibitors Tg, CPA, and BHQ also inhibit SPCA1α (24, 25). We confirmed that all compounds completely inhibit SERCA1α and SPCA1α, but we show that the compounds are effective in different concentration ranges for SPCA1α and SERCA1α (Table 1 and Fig. 3, C and D). For SERCA1α, the IC\(_{50}\) value of Tg falls below 10 nm, whereas higher (sub-\(\mu\)M) IC\(_{50}\) values are observed for CPA and BHQ. 2,2′-Methylenebis(6-tert-butyl-4-methylphenol) (bisphenol, BP) displays the lowest potency with an IC\(_{50}\) above 1 \(\mu\)M. For SPCA1α, the IC\(_{50}\) values of Tg and CPA are at least 3 orders of magnitude higher than for SERCA1α, whereas the IC\(_{50}\) value of BHQ is only 20 times higher. The IC\(_{50}\) value of BP is comparable for the two pumps, indicating that unlike what was suggested by a previous study (35), BP cannot be considered as a more selective inhibitor of SPCA1α than of SERCA1α. Remarkably, the slopes of the dose-response curves for BP (SERCA1α) and CPA (SPCA1α) are higher than 2 (2.9 ± 0.1 and 2.3 ± 0.1, respectively). This was also observed in earlier studies (25, 35) and may reflect differences in the cooperativity (37). In conclusion, the SERCA inhibitors Tg, CPA, and BHQ also inhibit SPCA1α, although at much higher concentrations.

Tg scaffold together with the O-2 moiety are mainly responsible for the inhibition of SPCA1α

Of the so-called specific SERCA inhibitors, Tg has the highest affinity for SPCA1α. Insights into the SAR of Tg on SPCA1α might be helpful to aid the development of Tg analogues with either low or high SPCA1α affinity. We therefore explored the SAR of Tg for SPCA1α and compared it with the well-established SAR of Tg for SERCA1α (38).

First, we compared the Tg-binding pocket in the SERCA1α-Tg structure and in an SPCA1α homology model in the same E2
Inhibition of SPCA1a by thapsigargin

Table 1
IC_{50} values for SPCA1a and SERCA1a measured for Tg, CPA, BHQ, and BP

|        | SPCA1a IC_{50} (μM) | SERCA1a IC_{50} (μM) |
|--------|----------------------|-----------------------|
| Tg     | 7.7 ± 1.5            | < 0.01                |
| CPA    | 182 ± 10             | 0.19 ± 0.01           |
| BHQ    | 13 ± 2               | 0.54 ± 0.06           |
| BP     | 3.5 ± 0.2            | 3.63 ± 0.09           |

conformation. In SERCA1a, the non-flexible tricyclic guaianolide nucleus of Tg provides a scaffold for the optimal position of side chains within the Tg-binding pocket formed by transmembrane helices M3, M5, and M7 (Fig. 4B). The lipophilic groups at O-3, C-4, O-8, and O-10 of Tg (Fig. 4A) critically determine the hydrophobic interaction and provide complementarity to the binding cavity in SERCA1a (39, 40). In the SERCA1a-Tg structure (41), the O-8 moiety of Tg orients to the interior of M3 and M5, whereas the O-2 chain is located in the interface between the transmembrane region and the membrane (42). By comparing the residues in the Tg-binding pocket of SERCA1a (Fig. 4B) and the homology model of SPCA1a (Fig. 4C), we anticipate that the Tg-binding affinity, mechanism of inhibition, and/or positioning of Tg are affected by several amino acid substitutions, including the homologous SERCA1a/SPCA1a

Only the O-2 moiety of Tg binds to a spatially restricted pocket in SPCA1a

In the further exploration of the poor contribution of the side chains at O-3, O-8, and O-10 to the affinity of Tg to SPCA1a, we considered the possibility that it might arise from a more spacious Tg-binding cavity than in SERCA, which in a more flexible way would accommodate these Tg side groups. This hypothesis was tested by replacing the O-2, O-3, O-8, and O-10 moieties with more bulky groups (Table 3). Replacing O-3 and O-10 with a slightly bulkier chain (HzL09012012 and HzL12012012, respectively) is well tolerated, whereas a more bulky biphenyl group on O-3 (HzL170809) or O-10 (HzL271009) led to a 7- and 49-fold higher IC_{50}, respectively (Table 3). However, the higher IC_{50} was significantly reduced after a longer pre-incubation time, indicating that a sufficiently large three-dimensional space is potentially available in the Tg pocket near O-3 and O-10 to allow accommodation of biphenyl groups (Table 3). The large but flexible Leu-8ADT substitution on O-8 led to a 19-fold reduction of the potency in SPCA1a most likely as a result of steric hindrance. However, overnight incubation of Leu-8ADT led to a lower IC_{50}, indicative of an induced fit effect and sufficient space to accommodate a larger O-8 chain (Table 3).

Of all tested Tg side groups, O-2 contributes most to the inhibition of SPCA1a. To determine the SAR of Tg in SPCA1a, we therefore focused on the role of O-2 by testing the functional impact of various substitutions of the O-2 group (Table 4). The IC_{50} remains unaffected when the O-2 octanoyl group was substituted with a benzoyl (HzL20072015) or naphthalene-carboxyl group (HzL18072015). Thus, the difference between HzL20072015, HzL18072015, and the −H substitution of nor-trilobolide (ΔO-2) indicates that the O-2 chain facilitates a hydrophobic interaction in the SPCA1a-binding pocket. However, a substitution of the O-2 moiety with a phenylbenzoyl group (HzL17072015) led to a significantly higher IC_{50} than with smaller substituents (HzL20072015 and HzL18072015), which was independent of the pre-incubation time. This reveals that the local three-dimensional space to accommodate a bulky O-2 group is restricted in SPCA1a, although the O-2 acyl group is larger in one dimension.

Therefore, we explored the replacement of the O-2 chain with several longer but flexible acyl groups (Table 4). We observed little effect on the IC_{50} by elongating the O-2 chain with two carbons (JBH04012016), whereas addition of four carbons (JBH09022016) increased the IC_{50} 3.4-fold (Table 4). Of interest, the inhibition of SPCA1a was completely abolished by introducing a more bulky end group (HzL24042015). The inhibitory effect of this compound is unaffected by the pre-incubation time.

Thus, strict spatial restrictions around O-2 are likely present, because only thin flexible O-2 chains are allowed, whereas more voluminous groups lower or prohibit the affinity for SPCA1a.
Inhibition of SPCA1a by thapsigargin

To explore the functional role of amino acid replacements in SPCA1a on Tg binding and confirm that Tg binds to the same pocket in SPCA1a and SERCA1a, we generated and purified three SPCA1a mutants in the Tg-binding pocket in which we substituted one or more of the SPCA1a-specific residues into the corresponding SERCA1a amino acids (L265F, Y272V, and L265F/Y272V/L776F) (Fig. 5A). Phe-256 is a critical residue for Tg central scaffold binding in SERCA1a (45) and is replaced in SPCA1a by Leu-265. The replacement of Val-263 in SERCA1a by Tyr-272 in SPCA1a introduces a bulky aromatic side chain possibly hindering Tg binding to SPCA1a. The Leu-776 in SPCA1a corresponds to Phe-834 in SERCA1a, which exerts unfavorable interaction with the O-10 group of Tg.

Compared with wild-type SPCA1a (WT), the more SERCA-like L265F and L265F/Y272V/L776F mutants are more sensitive to Tg, whereas Y272V behaves similarly as WT (Fig. 5B). This is consistent with the view that as in SERCA1a, Tg binds to a conserved pocket in the transmembrane region formed by M3, M5, and M7. Furthermore, the L265F substitution in SPCA1a significantly enhanced the binding affinity of Tg, possibly by promoting a direct interaction between Phe-265 and the Tg scaffold as is the case for SERCA1a (39). This result is also consistent with the view that binding of the Tg scaffold in SPCA1a may closely resemble the interaction in SERCA1a. Finally, the three SPCA1a mutants also displayed a more potent and stronger inhibition by CHEMS, a cholesterol analogue (Fig. 5C), suggesting that cholesteric compounds may bind at the same or overlapping site as Tg.

Modeling confirms the importance of the Tg scaffold in the binding to SPCA1a

To further explore how Tg might be accommodated in SPCA1a, we generated a homology model of SPCA1a and performed an in silico docking of Tg into the M3, M5, and M7 pocket, while allowing flexibility of 10 amino acid side chains in the Tg-binding pocket in which we substituted one or more of the SPCA1a-specific residues into the corresponding SERCA1a amino acids (L265F, Y272V, and L265F/Y272V/L776F) (Fig. 5A). Phe-256 is a critical residue for Tg central scaffold binding in SERCA1a (45) and is replaced in SPCA1a by Leu-265. The replacement of Val-263 in SERCA1a by Tyr-272 in SPCA1a introduces a bulky aromatic side chain in the Tg pocket possibly hindering Tg binding to SPCA1a. The Leu-776 in SPCA1a corresponds to Phe-834 in SERCA1a, which exerts unfavorable interaction with the O-10 group of Tg.

Compared with wild-type SPCA1a (WT), the more SERCA-like L265F and L265F/Y272V/L776F mutants are more sensitive to Tg, whereas Y272V behaves similarly as WT (Fig. 5B). This is consistent with the view that as in SERCA1a, Tg binds to a conserved pocket in the transmembrane region formed by M3, M5, and M7. Furthermore, the L265F substitution in SPCA1a significantly enhanced the binding affinity of Tg, possibly by promoting a direct interaction between Phe-265 and the Tg scaffold as is the case for SERCA1a (39). This result is also consistent with the view that binding of the Tg scaffold in SPCA1a may closely resemble the interaction in SERCA1a. Finally, the three SPCA1a mutants also displayed a more potent and stronger inhibition by CHEMS, a cholesterol analogue (Fig. 5C), suggesting that cholesteric compounds may bind at the same or overlapping site as Tg.
Inhibition of SPCA1α by thapsigargin

Table 3
IC₅₀ values of Tg analogues with modified O-3, O-8, or O-10 side chains on SPCA1α

| Compound | Structure | IC₅₀ SPCA (μM) |
|----------|-----------|---------------|
| Tg       | ![Structure](image) | 5.7 ± 1.5 |
| O-3 substitutions | | |
| HzL0912012 | ![Structure](image) | 6.7 ± 1.4 |
| HzL170699 | ![Structure](image) | 56 ± 10* |
| O-8 substitutions | | 28 ± 9 (ON) |
| Leu8ADT | ![Structure](image) | 143 ± 28* |
| 7 ± 3 (ON) | | |
| O-10 substitutions | | |
| HzL12012012 | ![Structure](image) | 10 ± 1 |
| HzL271009 | ![Structure](image) | 400 ± 100** |
| R° = | 47 ± 16 (ON)** |

Table 4
IC₅₀ values of Tg analogues with modified O-2 side chain on SPCA1α

| Compound | Structure | IC₅₀ SPCA (μM) |
|----------|-----------|---------------|
| Nootriboleide | R° = -H | 51 ± 1*** |
| HzL20072015 | ![Structure](image) | 57 ± 6**(ON) |
| HzL18072015 | ![Structure](image) | 12.5 ± 0.6 |
| HzL17072015 | ![Structure](image) | 13 ± 2 |
| JBBH040116 | ![Structure](image) | 37 ± 7*** |
| JBBH09022016 | ![Structure](image) | 12 ± 1 |
| HzL24092015 | ![Structure](image) | 26 ± 2* |
| No inhibition | | |

In this study, we purified for the first time the human Golgi/secretory pathway Ca²⁺/Mn²⁺-ATPase SPCA1α from a yeast overexpression model. The one-step affinity purification protocol generates hSPCA1α with over 95% purity offering opportunities for structure/function analysis and inhibitor screening. We demonstrate that SPCA1α is highly sensitive to the lipid environment and that several SERCA inhibitors, including Tg, also block SPCA1α activity, although at higher concentrations only. Because of the wide use of Tg in cellular studies and prospects for the use of Tg analogues in prostate cancer therapy, we focused on deciphering the SAR of Tg for SPCA1α. We observed differences in the relative contribution of Tg side chains in the inhibition of SPCA1α versus SERCA1α, which might be exploited for the future design of more selective SPCA1α or SERCA1α inhibitors.

Activity of SPCA1α depends on the membrane environment

Ca²⁺ transporters are tightly regulated by their lipid environment. For instance, the plasma membrane Ca²⁺-ATPase con-
tains two lipid-binding sites and can be stimulated by PS, PA, phosphatidylinositol bisphosphate, and cardiolipin (47). SERCA pumps are inhibited by sphingosine (48) and CL (49), whereas SPCA1a associates with CL-rich microdomains of human colon adenocarcinoma cells (30). Also, SPCA2 is inhibited by two endogenous signaling lipids oleamide and linoleamide (34). Here, we demonstrate that SPCA1a is inactive in a lipid-free detergent-solubilized state, but the activity restores upon reconstitution into a PC-rich lipid environment. In proteoliposomes, SPCA1a displays a higher apparent Ca$^{2+}$/H$^{1+}$ affinity and lower maximal ATPase activity than SERCA1a. The $K_m$ value of the purified and reconstituted SPCA1a falls within the
Inhibition of SPCA1a by thapsigargin

Table 5
Scores of the best Tg dockings in SPCA1a and SERCA1a

|            | SPCA1a | SERCA1a |
|------------|--------|---------|
| Docking score by | Fitness | Population | Gold.Chemscore | Fitness | Population |
| 30.22      | 1      | 31.68   | 1            |
| 27.92      | 2*     | 30.25   | 1            |
| 27.80      | 2*     | 30.21   | 1            |
| 27.57      | 2*     | 29.81   | 1            |
| 26.96      | 1      | 29.68   | 1            |
| 26.06      | 1      | 28.61   | 2            |
| 25.55      | 2*     | 28.58   | 2            |
| 24.99      | 2*     | 28.30   | 2            |
| 24.95      | 2*     | 28.19   | 2            |
| 24.87      | 2*     | 28.06   | 2            |

reported range for SPCA1 in cell membrane fractions (0.04–0.26 μM (8, 21, 50)). The variation in the reported K_m values presumably relates to slight differences in the free Ca^{2+} concentrations, the choice of the SPCA1 splice variant, and the selected cellular expression system, which may affect the regulation of SPCA1.

Moreover, SPCA1a is sensitive to the SERCA inhibitors Tg, CPA, and BHQ but also to bisphenol and CHEMS. SPCA1a was previously considered as Tg-insensitive, whereas a few studies reported a Tg sensitivity in the range between 0.1 and 30 μM (13, 24, 36). Here, we report that purified SPCA1a in reconstituted proteoliposomes displays an IC_{50} for Tg of 7.7 μM, which is four times lower than the reported IC_{50} value of 28 μM in microsomal fractions (24). The variations in the reported Tg sensitivities of SPCA1 may be due to differences in the experimental systems (membrane lipid composition and presence of other proteins or detergent). Thus, overall, the properties of the purified protein correspond well with SPCA1a in a cellular membrane context.

The inclusion of specific lipids in the proteoliposomes impacts on the activity of SPCA1a, with PA and SM leading to stimulation, whereas PE is inhibitory. PA is typically added during the reconstitution of SERCA1a to prevent aggregation (51). Because the stimulatory effect of PA was not observed with other charged lipids, it is also possible that PA serves as a regulatory lipid of SPCA1a. Although PA is a low-abundant lipid in the Golgi/secretory pathway, it can be locally formed by phospholipase D as a signaling lipid that may regulate SPCA1a activity (52, 53). Moreover, we showed that SPCA1a preferentially accumulates in lipid rafts (30), which is in line with the stimulatory effect of SM on the ATPase activity of SPCA1a. The cholesteric compound CHEMS5 only moderately inhibits SPCA1a, while exhibiting a more potent inhibitory effect on SERCA1a, in line with a different impact of CL on SPCA1a and SERCA1a as suggested previously (30). Although our reconstitution conditions were selected to promote the proper orientation of the protein in the membrane (i.e., the large hydrophilic cytosolic domain facing the outside of the vesicles) (51), we cannot fully rule out the possibility that the functional effects of the lipids may relate to an impact on the distribution between inward-facing and outward-facing enzymes in the proteoliposomes.

Of interest, the potency and efficacy of the CHEMS inhibition is higher in SPCA1a mutants with a more SERCA1a-like Tg pocket. The changes between the Tg-binding site of SERCA1a and SPCA1a might also explain the different sensitivity of SPCA1a and SERCA1a to cholesteric compounds. Although we cannot rule out that these mutations might exert an allosteric effect via an unrelated binding site for cholesteric compounds, it is reasonable to propose that the cholesteric compound binds and overlaps the Tg pocket. Indeed, it was already previously proposed that the SERCA1a Tg pocket might be a CL regulation site (39, 54). In the SERCA1a crystal structure 2EAU, the Tg-binding site was occupied by a lipid (55). Moreover, a fluorescence quenching study suggested the existence of hydrophobic non-annular binding sites for CL in SERCA1a (56), whereas a computational docking analysis modeled the steroid compound Alisol B, which targets SERCA pumps and enhances autophagy, in the Tg site (57). However, a more recent molecular dynamics simulation rather indicates that little CL may occupy the Tg-binding site (58), suggesting that the CL effect on SERCA1a may also be indirect via the impact on the membrane properties.

Inhibition of SPCA1a and SERCA1a by Tg is largely determined by the Tg scaffold

Of all tested pharmacological compounds, we observed the most potent inhibition of SPCA1a with Tg, a plant-derived sesquiterpene lactone that is generally accepted as a highly selective SERCA1a inhibitor. Over the years, structures of the Tg-SERCA1 complex (59–62), supported by extensive functional studies (38–40, 42, 43, 63–66), rendered a comprehensive pharmacophore model that explains the sub-nanomolar affinity of SERCA1a for Tg. The availability of purified SPCA1a and a library of Tg analogues now offered an opportunity to determine the SAR of Tg on SPCA1a, which might be helpful for the design of more specific SERCA1a or SPCA1a inhibitors.

In accordance with the view that Tg targets the same binding pocket in SPCA1a as in SERCA1a, amino acid substitutions in the predicted M3/M5 pocket of SPCA1a impact the affinity for Tg. According to our in silico docking, the Tg scaffold is aligned on the same plane irrespective of the side chain orientations. It was previously demonstrated that an F256L mutation in SERCA1a led to a 4-fold reduction in the Tg sensitivity (67), whereas our complementary experiment shows that the L265F substitution in SPCA1a significantly enhanced SPCA1a’s affinity for Tg. Thus, the Phe-256/Leu-265 substitution at least in SERCA1a significantly enhanced SPCA1a’s affinity for Tg. According to our in silico docking, the Tg scaffold is aligned on the same plane irrespective of the side chain orientations. It was previously demonstrated that an F256L mutation in SERCA1a led to a 4-fold reduction in the Tg sensitivity (67), whereas our complementary experiment shows that the L265F substitution in SPCA1a significantly enhanced SPCA1a’s affinity for Tg. Thus, the Phe-256/Leu-265 substitution at least in part explains the lower Tg affinity for SPCA1a than SERCA1a. The Tg scaffold might possibly undergo π-stacking with the L265F residue in the SPCA1a mutant. The importance of the Tg scaffold for inhibition is supported by findings with the 7,11-epoxide, which show a major reduction in the binding energy for both SPCA1a (Table 2) and SERCA1a (38, 39), pointing to possible similarities in the mechanism of inhibition in SPCA1a and SERCA1a.

Tg side groups differently contribute to the inhibition of SPCA1a versus SERCA1a

We further approached the SAR of Tg for SPCA1a by analyzing the IC_{50} values of a series of Tg analogues with modified side chains. Strikingly, removal of the O-3, O-8, and O-10 acyl groups have little effect on the binding energy of Tg in SPCA1a,
which is different for SERCA1a (Table 2) (40, 43). Moreover, substitution by slightly bulkier groups at these sites on SPCA1a is well tolerated, indicating that the Tg pocket in SERCA1a is spacious near O-3, O-8, and O-10 and that only few functional contacts exist between the side chains of Tg and residues of the protein. The removal of O-2, the longest side chain of Tg, leads to a higher IC₅₀ value in SPCA1a, suggesting that besides the relevance of the Tg scaffold, O-2 also contributes to the inhibition of SPCA1α. The analysis of Tg analogues with various O-2 substitutions further indicates that the O-2 chain is accommodated in a narrow hydrophobic cleft, which is restricted in volume. Noteworthy, the change in binding energy of the ΔO-2 compound is remarkably similar for SPCA1a and SERCA1a possibly pointing to a comparable binding mechanism.

By in silico docking analysis, two major docking solutions of Tg O-2 in SPCA1a were predicted. Of interest, the docking model 1 predicts a similar Tg orientation in SPCA1a as observed in the solved SERCA1a-Tg complex, which also might point to a similar inhibitory mechanism in SPCA1a and SERCA1a. This model may explain why a prolonged incubation of Leu-8ADT shows a more potent inhibition in SPCA1a (Table 3), because a similar effect of Leu-8ADT was observed on SERCA1a (44). Alternatively, in line with O-2 being accommodated into a tight hydrophobic cleft, the Tg O-2 chain in the SPCA1a model 2 protrudes into the M3/M5 region. Such a flipped orientation of Tg is also predicted for SERCA1a, although this is not experimentally observed in the solved SERCA1a-Tg complex, which may question the validity of such an orientation.

Toward the design of more specific SPCA1a inhibitors

We demonstrate that the SAR of Tg differs for SPCA1a and SERCA1a, which is mainly attributed to amino acid changes in the Tg pocket that prevent most interactions of the Tg side chains in SPCA1a, but it still allows the Tg scaffold to be accommodated in the pocket. The different SAR of Tg for SERCA1a and SPCA1a provides a strong rationale to consider the Tg scaffold as a lead compound for the development of more specific SPCA1a inhibitors. Solving the crystal structure of SPCA1a in the presence of Tg will greatly facilitate a rational design approach for a Tg-based SPCA1a inhibitor with higher potency and specificity, but so far, crystallization of SPCA1a turns out to be difficult in our hands.

In the absence of detailed structural information, we need to test additional Tg analogues with substitutions of the O-2, O-3, O-8, and O-10 chains to develop Tg analogues with a higher SPCA1a/SERCA1a specificity. Tg analogues with O-2 acyl chains that are slightly shorter would be interesting for establishing the optimal fit of O-2 within the SPCA1a Tg pocket. Moreover, our results indicate that single or combined bulky substitutions at O-3, O-8, and O-10 might lower the potency in SERCA1a, while having little impact on SPCA1a inhibition, favoring a higher SPCA1a/SERCA1a specificity. The more spacious environment near O-3 and O-10 indicates that bulkier side groups on both positions might lead to a tighter fit of Tg within the pocket.

A specific SPCA1a inhibitor might not only be a powerful tool to dissect the cellular and pathological role of SPCA1a in the Ca²⁺ (dys)homeostasis of various diseases, it might also be of therapeutic interest. Gain of SPCA1a and SPCA2 function is observed in breast cancer, because SPCA1a and/or SPCA2 proteins are up-regulated in various breast cancer sub-types contributing to a pathological Ca²⁺ dyshomeostasis (17, 18). Knockdown of endogenous SPCA2 prevents Orai1 activation, which reduces the transforming phenotype of MCF-7 cells in soft agar and impairs tumor generation upon injection in nude mice (18). Although this mainly depends on the direct interaction of SPCA2 with Orai1 leading to constitutive cytosolic Ca²⁺ influx (18), SPCA2 and Orai1 are likely mechanistically coupled, suggesting that also a higher Golgi Ca²⁺ uptake is contributing to the pathological Ca²⁺ dyshomeostasis (19). In line with this view, knockdown of SPCA1a in the basal-type breast cancer cell line MDA-MB-231 reduces, independent of Orai1, the processing of insulin-like growth factor receptor IGF1R (17), which might counteract its mitogenic and anti-apoptotic actions. Of further interest, the absence of PMR1, the SPCA1a orthologue in lower eukaryotes such as yeast, flies, and Caenorhabditis elegans, prevents the elevation of cytosolic Ca²⁺ and cell death caused by α-synuclein overexpression, a major toxic effector in Parkinson disease (68). Finally, SPCA1a mutations lead to a dominant skin disorder, Hailey-Hailey disease, which is generally considered to be caused by loss-of-function mutations that cause haploinsufficiency (15, 16). However, it was recently proposed that dominant disease mutations in ion pumps might introduce a gating defect resulting in ion leakage, which would represent a gain-of-function effect (69). In mice, heterozygous loss of SPCA1a does not trigger a similar skin disease, but it increases the risk of developing squamous cell tumors (70), further highlighting the importance of a balanced SPCA1a activity.

In conclusion, we demonstrate that Tg and cholesteric compounds in SPCA target an overlapping binding site on SPCA1a, and the differences in the SAR of Tg for SPCA1a and SERCA1a might allow the development of more specific inhibitors of SPCA1a.

Experimental procedures

Human SPCA1a expression in yeast

The recombinant human SPCA1a was cloned in the vector pTV001E with a C-terminal His₉ tag (CATCATCACAT-CACCATCACCAT) via Gateway cloning. Mutations were introduced via Q5 site-directed mutagenesis (New England Biolabs). pTV001-SPCA1a wild-type (WT) or mutant vectors were transformed into the S. cerevisiae strain BY4741a (his3Δ1; leu2Δ0;met15Δ0;ura3Δ0;MATa) as described previously (71). The yeast clone was inoculated in 10 ml of minimal medium (MM) deprived of Leu for overnight growth at 30 °C and gradually expanded into 2 liters MM-Leu culture over 3 days. On day 4, the yeast pellet was transferred into 25 liters of yeast peptone dextrose medium in a 50-liter Cell Bag (GE Healthcare), for 30 h of growth on a shaking system supplied with 30% O₂, 70% N₂ air mixture. Yeast cells were harvested by centrifugation (5,000 × g, 5 min, 4 °C) and washed with mil- li-Q water, and the pellets were weighted, flash-frozen, and stored at −20 °C.
Inhibition of SPCA1a by thapsigargin

Yeast membrane isolation

The yeast membrane was isolated based on the protocol described in Ref. 72. Briefly, the yeast pellet was suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.6 M sorbitol, 250 μM PMSE, 1 mM DTT, SIGMAFAST™ protease inhibitor mixture) and subjected twice to high pressure homogenization at 140,000 kPa (High Pressure Homogenizer, Avestin). The membrane fractions were collected by differential centrifugation at 1,000 and 16,000 g, and they were suspended in solubilization buffer (10 mM imidazole, pH 7.5, 50 mM NaH2PO4, pH 7.5, 500 mM NaCl, 20% glycerol, 250 μM PMSE, 1 mM DTT, SIGMAFAST™). Protein concentration was determined by the Bradford assay. The yeast membrane fraction was aliquoted, flash-frozen in liquid N2, and stored at −80 °C.

Purification of SPCA1a and SERCA1a by affinity chromatography

SPCA1a was purified from rabbit fast twitch skeletal muscle as described before (29), whereas a purification protocol for SERCA1a was optimized as detailed below. The His6-tagged SPCA1a was purified via nickel-affinity chromatography using nickel-charged agarose beads coupled to Ni-NTA. One column volume (CV) of nickel-Sepharose high performance beads (GE Healthcare) was pre-equilibrated with 6 CV of equilibration buffer (50 mM NaH2PO4, pH 7.5, 75 mM imidazole, pH 7.5, 500 mM NaCl, 20% glycerol, 5 mM 2-mercaptoethanol (2-ME), SIGMAFAST™ protease inhibitor mixture, 5 mM 2-ME), and non-soluble material was removed by ultracentrifugation (160,000 × g, 45 min, 4 °C). After binding of the supernatant to the column for 1 h at 4 °C, the liquid was drained off. The beads were washed with 6 CV of three consecutive washing buffers composed of binding buffer with a stepwise reduction in the NaCl and DDM concentrations: from 500 to 50 and 50 mM NaCl combined with 5, 1, and 0.25 mg/ml DDM, respectively. SPCA1a was eluted in the final equilibration buffer with 50 mM NaCl and 0.25 mg/ml DDM. The washing and elution buffers were supplemented with PC (Avanti Polar Lipids) at the ratio 1:0.67 (DDM/PC). Afterward, the buffer component of purified SPCA1a was exchanged into 20 mM imidazole, pH 7.5, 100 mM KCl, 20% glycerol, 0.25 mg/ml DDM, 0.167 mg/ml PC, 5 mM 2-ME, SIGMAFAST™ via PD Mantrap G-25 (GE Healthcare) column. Aggregates were removed by centrifugation at 20,000 × g for 20 min at 4 °C. The supernatant was collected and flash-frozen in liquid N2, and stored at −80 °C. The purified SPCA1a was analyzed by MALDI-TOF mass spectrometry (4800 Proteomics Analyzer, AB Sciex) in the linear ion mode in the presence of α-cyano-4-hydroxycinnamic acid (5 mg/ml).

Reconstitution of the SPCA1a and SERCA1a Ca2+-ATPases

The 550-μg lipid mixture dissolved with chloroform in a glass tube was dried in a vacuum desiccator under a N2 stream and overnight in a vacuum desiccator. Liposome buffer (150 μl of 20 mM imidazole, pH 7, 100 mM NaCl, 10 mM MgCl2, and 1 mM DTT) was added and incubated at 37 °C water bath for 1 h with short vortex periods in between. To this (unless otherwise specified) 30 μg of Triton X-100 was added at room temperature, and the mixture was vortexed for 3 min followed by addition of purified SPCA1a (200 μg of protein at a concentration 0.667 mg/ml). Detergent was extracted to form proteoliposomes by treatment with excess Bio-Beads SM-2 (Bio-Rad) for 2.5 h.

The proteoliposomes were purified by sucrose gradient centrifugation (20 and 50% sucrose in 20 mM imidazole, pH 7, 0.2% azide, 100 mM NaCl) at 150,000 × g, 75 min, and 4 °C from which a 450-μl proteoliposome fraction was collected from the turbid layer present between the two sucrose fractions. This sample was flash-frozen in liquid N2 and stored at −80 °C. The reconstitution of SPCA1a was performed according to Ref. 29. PC, PA, and PE from chicken egg, PS and SM from porcine brain, and phosphatidylinositol from bovine liver were purchased from Avanti Polar Lipids, whereas CL was purchased from Sigma.

Protein electrophoresis and immunoblotting

Purified or reconstituted SPCA1a or SERCA1a was separated on NuPAGE 4–12% Bis-Tris polyacrylamide gel (200 V, 35 min, MES buffer) (Thermo Fisher Scientific) and visualized with Imperial Protein Stain (Thermo Fisher Scientific). SeeBlue Plus2 pre-stained protein standard (Thermo Fisher Scientific) was used as a molecular weight marker. The concentration of purified protein was determined on a denaturing gel by comparison with 0.125 to 2 μg/μl bovine serum albumin (BSA) standards (BSA Standard Ampules, Pierce). For immunoblotting, primary antibodies against the His tag (1:2,000 dilution, penta-His, Qiagen) or SPCA1a (1:50,000 dilution, Frodo, homemade) were used. Horseradish peroxidase-linked secondary antibodies (Cell Signaling Technology) were used for enhanced chemiluminescent detection (Bio-Rad ChemiDoc). MagicMark (Thermo Fisher Scientific) was used as the molecular weight marker.

ATPase assay

The Ca2+-dependent ATPase activity assay was performed on reconstituted SPCA1a or SERCA1a samples according to the Baginsky protocol as described previously (73). The reactions were performed for 15 min (SERCA1a, 150 ng in 50 μl) or 30 min SPCA1a (300 ng in 50 μl). The results were fitted with a Hill function.

The dose response of the ATPase to various compounds was performed by measuring the ATPase activity at 1 μM free Ca2+ concentration with increasing concentrations of compounds to be tested in the reaction mix. Reactions with Tg (200 nm for SERCA1a and 500 μM for SPCA1a) samples were used as blanks to correct for irrelevant color development. The data were normalized as the percentage of inhibition compared with that obtained in the absence of inhibitor and fitted with a logistic curve. The CHEMS, CPA, BHQ, BP, and oleamide were purchased from Sigma. Linoleamide was purchased from Enzo Life Sciences, Tg was purchased from Alomone Labs. All compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma).

In silico modeling

The SPCA1a homology model was generated with the homology model module in MOE 2014.09 (74) based on the
SERCA1a E2 crystal structure with Tg bound (PDB code 1IWO (41)). Subsequently, the structure of the homology model was energy-minimized using Amber99 (75) force field in the energy minimization module of MOE. Tg was docked in the SERCA1a homology model or SERCA1a crystal structure via GOLD version 5.2 (Genetic Optimization for Ligand Docking (76, 77)). Tg was docked in the SERCA1a E2 crystal structure without any inhibitors (PDB code 3W5C (46) and compared with the crystal structure 1IWO with Tg bound. We specified the center of the Tg-binding site (x, 33.422; y, −18.503; and z, 82.4683) and selected all atoms in a radius of 11 Å. 10 residues in the Tg-binding site (Glu-255, Phe-256, Gln-259, Leu-260, Val-263, Ile-272, Leu-707, Ile-711, Ile-770, Leu-771, Ile-776, and Ile-779) were selected to allow for receptor flexibility in a radius of 10 Å. 10 residues in the binding site (Leu-264, Leu-269, Tyr-272, Leu-707, Ile-711, Ile-770, Leu-771, Ile-776, and Ile-779) were selected to allow for receptor flexibility through side-chain torsion rotations. For each of these residues, flexibility was limited to a library of selected side chain conformers extracted from published crystal structures. Docking conformations for Tg were generated by performing 20 runs using standard Genetic Algorithm parameters. The docking results were scored and sorted using the Chemscore scoring function of GOLD.

The docking poses for Tg or the analogues were generated in the SERCA1a E2 homology model using the GOLD version 5.2 docking software. Here, we specified the center of the binding site (x, −2.8375; y, −22.0812; and z, 12.2) and selected all atoms in a radius of 10 Å. 10 residues in the binding site (Leu-264, Leu-269, Tyr-272, Leu-707, Ile-711, Ile-770, Leu-771, Ile-776, and Ile-779) were selected to allow for receptor flexibility through side-chain torsion rotations. To generate docking conformations, 100 runs were performed for each ligand using Genetic Algorithm parameters. The docking results were scored and sorted using the Chemscore scoring function of GOLD.

**Synthesis of Tg analogues**

The synthesis of the Tg analogues is described in Supplemental Materials and methods.

**Statistical analysis**

Values are provided as mean ± S.E. Hill or logistic fitting was performed via OriginPro 9 software. Statistical significance was calculated by one-way t test and indicated by asterisks: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

**Author contributions**—P. V. conceived the idea for the project and coordinated the study. J. C. conducted most of the experiments and analyzed the data. J. D. R., M. D. M., J. B. H., S. S., and I. V. conducted experiments on in silico modeling. Tg analogue synthesis, and SPCA1a purification. J. V. M., J. E., S. B. C., P. V., and F. W. analyzed the data and critically reviewed the manuscript. P. V. and J. C., wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

**References**

1. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Calcium signalling: dynamics, homeostasis and remodelling. Nat. Rev. Mol. Cell Biol. 4, 517–529

2. Monteith, G. R., McAndrew, D., Faddy, H. M., and Roberts-Thomson, S. J. (2007) Calcium and cancer: targeting Ca2+ transport. Nat. Rev. Cancer 7, 519–530

3. Palmgren, M. G., and Nissen, P. (2011) P-type ATPases. Annu. Rev. Biochem. 80, 243–266

4. Vangheluwe, P., Sepidveda, M. R., Missiaen, L., Raeymaekers, L., Wuytack, F., and Vanoevelen, J. (2009) Intracellular Ca2+2- and Mn2+2-transport ATPases. Chem. Rev. 109, 4733–4759

5. Behne, M. J., Tu, C. L., Aronchik, I., Epstein, E., Bench, G., Bikle, D. D., Pozzan, T., and Mauro, T. M. (2003) Human keratinocyte ATP2C1 localizes to the Golgi and controls Golgi Ca2+ stores. J. Invest. Dermatol. 121, 688–694

6. Xiang, M., Mohamalawari, D., and Rao, R. (2005) A novel isoform of the secretory pathway Ca2+/Mn2+-ATPase, hSPCA2, has unusual properties and is expressed in the brain. J. Biol. Chem. 280, 11608–11614

7. Wuytack, F., Raeymaekers, L., and Missiaen, L. (2002) Molecular physiology of the SERCA and SPCA pumps. Cell Calcium 32, 279–305

8. Ton, V. K., Mandal, D., Vahadji, C., and Rao, R. (2002) Functional expression in yeast of the human secretory pathway Ca2+/Mn2+-ATPase defective in Hailey-Hailey disease. J. Biol. Chem. 277, 6422–6427

9. Mitchell, K. J., Tsuoi, T., and Rutter, G. A. (2004) Role for plasma membrane-related Ca2+-ATPase-1 (ATP2C1) in pancreatic beta-cell Ca2+ homeostasis revealed by RNA silencing. Diabetes 53, 399–400

10. Vanoevelen, J., Vanoevelen, J., Callewaert, G., Parys, J. B., De Smedt, H., Raeymaekers, L., Ruzzuto, R., Missiaen, L., and Wuytack, F. (2003) The contribution of the SPCA1 Ca2+ pump to the Ca2+ accumulation in the Golgi apparatus of HeLa cells assessed via RNA-mediated interference. Biochim. Biophys. Res. Commun. 306, 430–436

11. Micaroni, M., Giaichetti, G., Plebani, R., Xiao, G. G., and Federici, L. (2016) ATP2C1 gene mutations in Hailey-Hailey disease and possible roles of SPCA1 isoforms in membrane trafficking. Cell Death Dis. 7, e2259

12. Vanoevelen, J., Dode, L., Van Baelen, K., Fairclough, R. J., Missiaen, L., Raeymaekers, L., and Wuytack, F. (2005) The secretory pathway Ca2+/Mn2+-ATPase 2 is a Golgi-localized pump with high affinity for Ca2+ ions. J. Biol. Chem. 280, 22880–22888

13. Baron, S., Struyf, S., Wuytack, F., Van Damme, J., Missiaen, L., Raeymaekers, L., and Vanoevelen, J. (2009) Contribution of intracellular Ca2+ stores to Ca2+ signaling during chemokinesis of human neutrophil granulocytes. Biochim. Biophys. Acta 1793, 1041–1049

14. Faddy, H. M., Smart, C. E., Xu, R., Lee, G. Y., Kenny, P. A., Feng, M., Rao, R., Brown, M. A., Bissell, M. J., Roberts-Thomson, S. J., and Monteith, G. R. (2008) Localization of plasma membrane and secretory calcium pumps in the mammary gland. Biochem. Biophys. Res. Commun. 369, 977–981

15. Missiaen, L., Raeymaekers, L., Dode, L., Vanoevelen, J., Van Baelen, K., Parys, J. B., Callewaert, G., De Smedt, H., Segaert, S., and Wuytack, F. (2004) SPCA1 pumps and Hailey-Hailey disease. Biochem. Biophys. Res. Commun. 322, 1204–1213

16. Burge, S. M. (1992) Hailey-Hailey disease: the clinical features, response to treatment and prognosis. Br. J. Dermatol. 126, 275–282

17. Grice, D. M., Vetter, I., Faddy, H. M., Kenny, P. A., Roberts-Thomson, S. J., and Monteith, G. R. (2010) Golgi calcium pump secretory pathway calcium ATPase 1 (SPCA1) is a key regulator of insulin-like growth factor receptor (IGFIR) processing in the basal-like breast cancer cell line MDA-MB-231. J. Biol. Chem. 285, 37458–37466

18. Feng, M., Grice, D. M., Faddy, H. M., Nguyen, N., Leitch, S., Wang, Y., Muend, S., Kenny, P. A., Sukumar, S., Roberts-Thomson, S. J., Monteith, G. R., and Rao, R. (2010) Store-independent activation of Orai1 by SPCA2 in mammary tumors. Cell 143, 84–98

19. Smaardijk, S., Chen, J., Wuytack, F., and Vangheluwe, P. (2016) SPCA2 couples Ca2+ influx via Orai1 to Ca2+ uptake into the Golgi/secretory pathway. Tissue Cell 2016, S0040

20. Cross, B. M., Hack, A., Reinhardt, T. A., and Rao, R. (2013) SPCA2 regulates Orai1 trafficking and store independent Ca2+ entry in a model of lactation. PLoS ONE 8, e67348

21. Dode, L., Andersen, J. P., Raeymaekers, L., Missiaen, L., Vilsen, B., and Wuytack, F. (2005) Functional comparison between secretory pathway Ca2+/Mn2+-ATPase (SPCA1) and sarcoplasmic reticulum Ca2+-ATPase (SERCA1) isoforms by steady-state and transient kinetic analyses. J. Biol. Chem. 280, 39124–39134

22. Michelangeli, F., and East, J. M. (2011) A diversity of SERCA Ca2+ pump inhibitors. Biochem. Soc. Trans. 39, 789–797
Inhibition of SPCA1α by thapsigargin

23. Sorin, A., Rosas, G., and Rao, R. (1997) PMR1, a Ca^{2+}-ATPase in yeast Golgi, has properties distinct from sarco/endoplasmic reticulum and plasma membrane calcium pumps. J. Biol. Chem. 272, 9895–9901
24. Dode, L., Andersen, J. P., Vanoevelen, J., Raeymaekers, L., Missiaen, L., Vilsen, B., and Wuytack, F. (2006) Dissection of the functional differences between human secretory pathway Ca^{2+}/Mn^{2+}-ATPase (SPCA1) and 2 isoforms by steady-state and transient kinetic analyses. J. Biol. Chem. 281, 3182–3189
25. Missiaen, L., Vanoevelen, J., Parys, J. B., Raeymaekers, L., De Smidt, H., Callewaert, G., Erneux, C., and Wuytack, F. (2002) Ca^{2+} uptake and release properties of a thapsigargin-insensitive nonmitochondrial Ca^{2+} store in A7r5 and 16HBE14o– cells. J. Biol. Chem. 277, 6898–6902
26. Denmeade, S. R., Mhaka, A. M., Rosen, D. M., Brennen, W. N., Dalrymple, S., Dach, I., Olesen, C., Gurel, B., Demarzo, A. M., Wilding, G., Carducci, M. A., Dionne, C. A., Møller, J. V., Nissen, P., Christensen, S. B., and Isacs, J. T. (2012) Engineering a prostate-specific membrane antigen-activated tumor endothelial cell prodrug for cancer therapy. Sci. Transl. Med. 4, 140ra186
27. Doan, N. T., Paulsen, E. S., Sehgal, P., Møller, J. V., Nissen, P., Denmeade, S. R., Isacs, J. T., Dionne, C. A., and Christensen, S. B. (2015) Targeting thapsigargin toward tumors. Steroids 97, 2–7
28. Rigaud, J. L. (2002) Membrane proteins: functional and structural studies using reconstituted proteoliposomes and 2-D crystals. Braz. J. Med. Res. 35, 753–766
29. Young, H. S., Rigaud, J. L., Lacape`re, J. J., Reddy, L. G., and Stokes, D. L. (1997) How to make tubular crystals by reconstitution of detergent-solu- bilized Ca^{2+}-ATPase. Biophys. J. 72, 2545–2558
30. Baron, S., Vangheluwe, P., Sepulveda, M. R., Wuytack, F., Raeymaekers, L., and Vanoevelen, J. (2010) The secretory pathway Ca^{2+}-ATPase 1 is associated with cholesterol-rich microdomains of human colon adenocarci-noma cells. Biochim. Biophys. Acta 1798, 1512–1521
31. Kadowaki, H., Grant, M. A., and Seyfried, T. N. (1994) Effect of Golgi membrane phospholipid composition on the molecular species of GM3 gangliosides synthesized by rat liver sialyltransferase. J. Lipid Res. 35, 1956–1964
32. van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave. Nat. Rev. Mol. Cell Biol. 9, 112–124
33. Lambert, O., Levy, D., Ranck, J. L., Leblanc, G., and Rigaud, J. L. (1998) A new “gel-like” phase in dodecyl maltoside-lipid mixtures: implications in solubilization and reconstitution studies. Biophys. J. 74, 918–930
34. Yamamoto, S., Takeda, M., Kabashima, Y., Fukutomi, T., and Ushimaru, M. (2016) Identification of novel inhibitors of human SPCA2. Biochem. Biophys. Res. Commun. 477, 266–270
35. Lai, P., and Michelangeli, F. (2012) Bis(2-hydroxy-3-tert-butyl-5-methyl-phenyl)-methane (bis-phenol) is a potent and selective inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) Ca^{2+} pump isoforms 1, 2, and 3 to thapsigargin and other inhibitors. J. Biol. Chem. 281, 6970–6976
36. Toyoshima, C., Iwasawa, S., Ogawa, H., Hirata, A., Tsuda, J., and Inesi, G. (2013) Crystal structures of the calcium pump and sarcolin in the Mg^{2+}-bound El state. Nature 495, 260–264
37. Streher, E. E., and Zacharias, D. A. (2001) Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. Physiol. Rev. 81, 21–50
38. Benaim, G., Pimentel, A. A., Felibert, P., Mayora, A., Colman, L., Sojo, F., Rojas, H., and De Sanctis, J. B. (2016) Sphingosine inhibits the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) activity. Biochem. Biophys. Res. Commun. 473, 572–577
39. Li, Y., Ge, M., Ciani, L., Kuriakose, G., Westover, E. J., Dura, M., Covey, D. F., Freed, J. H., Maxfield, F. R., Lytton, J., and Tabas, I. (2004) Enrichment of endoplasmic reticulum with cholesterol inhibits sarco- plasmic-endoplasmic reticulum calcium ATPase-2b activity in parallel with increased order of membrane lipids: implications for depletion of endoplasmic reticulum calcium stores and apoptosis in cholesterol-loaded macrophages. J. Biol. Chem. 279, 37030–37039
40. Fairclough, R. J., Dode, L., Vanoevelen, J., Andersen, J. P., Missiaen, L., Raeymaekers, L., Wuytack, F., and Hovnanian, A. (2003) Effect of Hailey- Hailey disease mutations on the function of a new variant of human secretory pathway Ca^{2+}/Mn^{2+}-ATPase (hSPCA1). J. Biol. Chem. 278, 24721–24730
41. Rigaud, J. L., and Lévy, D. (2003) Reconstitution of membrane proteins into liposomes. Methods Enzymol. 372, 65–86
42. Selvy, P. E., Lavieri, R. R., Lindsay, C. W., and Brown, H. A. (2011) Phospholipase D: enzymology, functionality, and chemical modulation. Chem. Rev. 111, 6064–6119
43. Holemans, T., Sørensen, D. M., van Veen, S., Martin, S., Hermans, D., Kemmer, G. C., Van den Haute, C., Baekelandt, V., Gümüther Pomorski, T., Agostinis, P., Wuytack, F., Palmgren, M., Eggermont, J., and Vangheluwe, J. (2015) A lipid switch unlocks Parkinson’s disease-associated ATP13A2. Proc. Natl. Acad. Sci. U.S.A. 112, 9040–9045
44. Lee, A. G. (2003) Lipid-protein interactions in biological membranes: a structural perspective. Biochim. Biophys. Acta 1612, 1–40
45. Takahashi, M., Kondou, Y., and Toyoshima, C. (2007) Interdomain communication in calcium pump as revealed in the crystal structures with transmembrane inhibitors. Proc. Natl. Acad. Sci. U.S.A. 104, 5800–5805
46. Simmonds, A. C., East, J. M., Jones, O. T., Rooney, E. K., McWhirter, J., and Lee, A. G. (1992) Annuar and non-annular binding sites on the (Ca^{2+} + Mg^{2+})-ATPase. Biochim. Biophys. Acta 963, 398–406
47. Law, B. Y., Wang, M., Ma, D. L., Al-Mousa, F., Michelangeli, F., Cheng, S. H., Ng, M. H., To, K. F., Mok, A. Y., Ko, R. Y., Lam, S. K., Chen, F., Che, C. M., Chiu, P., and Ko, B. C. (2010) Alisol B, a novel inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump, induces autophagy, endoplasmic reticulum stress, and apoptosis. Mol. Cancer Ther. 9, 718–730
48. Autzen, H. E., Siuda, I., Sonntag, Y., Nissen, P., Møller, J. V., and Thogersen, L. (2015) Regulation of the Ca^{2+} -ATPase by cholesterol: a specific or non-specific effect? Mol. Membr. Biol. 32, 75–87

**References:**
6950
Inhibition of SPCA1a by thapsigargin

59. Jensen, A. M., Sørensen, T. L., Olesen, C., Møller, J. V., and Nissen, P. (2006) Modulatory and catalytic modes of ATP binding by the calcium pump. J. Biol. Chem. 281, 11303–11314

60. Obara, K., Miyashita, N., Xu, C., Toyoshima, I., Sugita, Y., Inesi, G., and Toyoshima, C. (2005) Structural role of countertransport revealed in Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. USA. 102, 14489–14496

61. Toyoshima, C., Norimatsu, Y., Iwasawa, S., Tsuda, T., and Ogawa, H. (2007) How processing of aspartylphosphate is coupled to luminal gating of the ion pathway in the calcium pump. Proc. Natl. Acad. Sci. USA. 104, 19831–19836

62. Toyoshima, C., Yonekura, S., Tsueda, J., and Iwasawa, S. (2011) Trinitrophenyl derivatives bind differently from parent adenine nucleotides to Ca^{2+}-ATPase in the absence of Ca^{2+}. Proc. Natl. Acad. Sci. USA. 108, 1833–1838

63. Andersen, A., Cornett, C., Lauridsen, A., Olsen, C. E., and Christensen, S. B. (1994) Selective transformations of the Ca^{2+}-pump inhibitor thapsigargin. Acta Chem. Scand. 48, 340–346

64. Smitt, U. W., and Christensen, S. B. (1991) Nortrilobolide, a new potent guaianolide secretagogue from Thapsia garganica. Planta Med. 57, 196–197

65. Xu, C., Ma, H., Inesi, G., Al-Shawi, M. K., and Toyoshima, C. (2004) Specific structural requirements for the inhibitory effect of thapsigargin on the Ca^{2+}-ATPase SERCA. J. Biol. Chem. 279, 17973–17979

66. Yu, M., Lin, J., Khadeer, M., Yeh, Y., Inesi, G., and Hussain, A. (1999) Effects of various amino acid 256 mutations on sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase function and their role in the cellular adaptive response to thapsigargin. Arch. Biochem. Biophys. 362, 225–232

67. Yu, M., Zhong, L., Rishi, A. K., Khadeer, M., Inesi, G., Hussain, A., and Zhang, L. (1998) Specific substitutions at amino acid 256 of the sarcoplasmic/endoplasmic reticulum Ca^{2+} transport ATPase mediate resistance to thapsigargin in thapsigargin-resistant hamster cells. J. Biol. Chem. 273, 3542–3546

68. Büttrner, S., Faes, L., Reichelt, W. N., Broeskamp, F., Habernig, L., Benke, S., Kourits, N., Ruli, D., Carmona-Gutierrez, D., Eisenberg, T., D’hooge, P., Ghiillebert, R., Franssens, V., Harger, A., Pieber, T. R., et al. (2013) The Ca^{2+}/Mn^{2+} ion-pump PMR1 links elevation of cytosolic Ca^{2+} levels to α-synuclein toxicity in Parkinson’s disease models. Cell Death Differ. 20, 465–477

69. Kaneko, M., Desai, B. S., and Cook, B. (2014) Ionic leakage underlies a gain-of-function effect of dominant disease mutations affecting diverse P-type ATPases. Nat. Genet. 46, 144–151

70. Okunade, G. W., Miller, M. L., Azhar, M., Andringa, A., Sanford, L. P., Doetschman, T., Prasad, V., and Shull, G. E. (2007) Loss of the ATP2C1 secretory pathway Ca^{2+}-ATPase (SPCA1) in mice causes Golgi stress, apoptosis, and midgestational death in homozygous embryos and squamous cell tumors in adult heterozygotes. J. Biol. Chem. 282, 26517–26527

71. Vandecaetsbeek, I., Christensen, S. B., Liu, H., Van Veldhoven, P. P., Waelkens, E., Eggermont, J., Raeymaekers, L., Møller, J. V., Nissen, P., Wuytack, F., and Vangheluwe, P. (2011) Thapsigargin affinity purification of intracellular P(2A)-type Ca^{2+} ATPases. Biochim. Biophys. Acta 1813, 1118–1127

72. Iidenko, M., Lenoir, G., Fuentes, J. M., le Maire, M., and Jaxel, C. (2006) Expression in yeast and purification of a membrane protein, SERCA1a, using a biotinylated acceptor domain. Protein Expr. Purif. 48, 32–42

73. Holemans, T., Vandecaetsbeek, I., Wuytack, F., and Vangheluwe, P. (2014) Measuring Ca^{2+} pump activity in overexpression systems and cardiac muscle preparations. Cold Spring Harb Protoc 2014, 876–886

74. Chemical Computing Group Inc. (2014) Molecular Operating Environment (MOE), version 2014.09, Chemical Computing Group Inc., Montreal, Quebec, Canada

75. Wang, J. M., Cieplak, P., and Kollman, P. A. (2000) How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? J. Comput. Chem. 21, 1049–1074

76. Jones, G., Willett, P., and Glen, R. C. (1995) Molecular recognition of receptor-sites using a genetic algorithm with a description of desolvation. J. Mol. Biol. 245, 43–53

77. Jones, G., Willett, P., Glen, R. C., Leach, A. R., and Taylor, R. (1997) Development and validation of a genetic algorithm for flexible docking. J. Mol. Biol. 267, 727–748