Published in final edited form as:

Biochem Biophys Res Commun. 2016 December 9; 481(3-4): 206–211. doi:10.1016/j.bbrc.2016.10.096.

Direct detection of SERCA calcium transport and small-molecule inhibition in giant unilamellar vesicles

Tengfei Bian^{a,b}, Joseph M. Autry^{c,d}, Denise Casemore^a, Ji Li^{c,d}, David D. Thomas^{c,1}, Gaohong He^{b,1}, and Chengguo Xing^{a,*,1,2}

^aDepartment of Medicinal Chemistry, College of Pharmacy, University of Minnesota, 2231 6th St SE, Minneapolis, MN 55455, United States

^bState Key Laboratory of Fine Chemicals, R&D Center of Membrane Science and Technology, Dalian University of Technology, Dalian 116024, China

^cDepartment of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, 321 Church St SE, Minneapolis, MN 55455, United States

^dBiophysical Technology Center, Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, 321 Church St SE, Minneapolis, MN 55455, United States

Abstract

We have developed a charge-mediated fusion method to reconstitute the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) in giant unilamellar vesicles (GUV). Intracellular Ca^{2+} transport by SERCA controls key processes in human cells, such as proliferation, signaling, and contraction. Small-molecule effectors of SERCA are urgently needed as therapeutics for Ca^{2+} dysregulation in human diseases including cancer, diabetes, and heart failure. Here we report the development of a method for efficiently reconstituting SERCA in GUV, and we describe a streamlined protocol based on optimized parameters (e.g., lipid components, SERCA preparation, and activity assay requirements). ATP-dependent Ca^{2+} transport by SERCA in single GUV was detected directly using confocal fluorescence microscopy with the Ca^{2+} indicator Fluo-5F. The GUV reconstitution system was validated for functional screening of Ca^{2+} transport using thapsigargin (TG), a small-molecule inhibitor of SERCA currently in clinical trials as a prostate cancer prodrug. The GUV system overcomes the problem of inhibitory Ca^{2+} accumulation for SERCA in native and reconstituted small unilamellar vesicles (SUV). We propose that charge-mediated fusion provides a widely-applicable method for GUV reconstitution of clinically-important membrane transport proteins. We conclude that GUV reconstitution is a technological advancement for evaluating small-molecule effectors of SERCA.

*Corresponding author: Chengguo Xing. chengguoxingcop.ufl.edu. Fax: +1 (352) 273-9724.

¹Co-senior authors: David D. Thomas, Gaohong He, and Chengguo Xing.

²Present address: Chengguo Xing, University of Florida, College of Pharmacy, Department of Medicinal Chemistry, 1345 Center Drive, Gainesville, FL 32610, United States

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Appendix A. Supplemental Data

Supplemental Material associated with this article can be found in the online version at BBRC.

Graphical abstract

Keywords

biomimetic membrane; calcium regulation; drug discovery; electrostatic fusion; lipid reconstitution; transport proteins

1. Introduction

SERCA is a 110-kDa membrane transport protein that maintains low (submicromolar) cytoplasmic Ca²⁺ in human cells. SERCA uses the energy of ATP hydrolysis to pump Ca²⁺ from the cytosol into the endoplasmic reticulum (ER) of non-muscle cells and into the sarcoplasmic reticulum (SR) of myocytes [1,2]. Ca²⁺ is a central messenger that controls key cellular functions; for example, proliferation, signaling, contractility, metabolism, transcription, protein folding, and apoptosis. Ca²⁺ dysregulation is associated with numerous disorders, including cardiovascular and muscular diseases, and multi-drug resistant cancers [3,4]. Small-molecule compounds that activate SERCA have been effective against cell and animal models of degenerative disease such as heart failure, diabetes, and Alzheimer's [5,6,7,8]. Conversely, small-molecule compounds that inhibit SERCA have been effective against models of proliferative disease such as cancer and parasitic infection [9,10]. Thus, SERCA is an important therapeutic target, yet SERCA-based pharmacological therapy has not yet reached the clinic.

In the quest for SERCA drug discovery, a direct and accurate assay of Ca²⁺ transport remains an outstanding problem. Standard preparations for measuring SERCA activity and regulation are native SR vesicles (0.2–0.4 μm in diameter) isolated from and reconstituted SUV (0.1–0.2 μm in diameter) with synthetic lipids and purified SERCA [11,12]. Ca²⁺ transport into these preparations is typically measured using radioactive ⁴⁵Ca²⁺ (filtration assay) or a Ca²⁺-sensitive dye (optical assay) [13,14]. However, it is not feasible to measure Ca²⁺ transport directly in these preparations, because internal Ca²⁺ concentration reaches inhibitory levels (0.1–1 mM) rapidly: < 1 second for SR vesicles and < 1 minute for SUV reconstituted at very high lipid/SERCA ratio (mol/mol) [11,12]. Therefore, anionic Ca²⁺ precipitants with low solubility constant (such as phosphate, oxalate, or citrate) are typically added at 5–100 mM concentration inside native vesicles and SUV proteoliposomes, which causes intravesicular Ca²⁺ to precipitate at relatively low luminal concentration and thus allow extended assay of steady-state Ca²⁺ transport for 1–10 min [15,16]. However, Ca²⁺ precipitants cause significant inhibition of SERCA and ultimately result in membrane disruption through crystal growth, thereby limiting the use of steady-state enzyme assays.

GUV is an ideal system to study membrane transport, because GUV avoid the problem of product inhibition intrinsic to native vesicles and reconstituted SUV, i.e., GUV with a diameter of 20 μm have a volume 1,000,000-fold greater than the volume of SUV with diameter of 0.2 μm . To date, a few methods have been successfully used to incorporate membrane proteins in GUV, such as direct electroformation, detergent-mediated reconstitution, and peptide-mediated GUV–SUV fusion [17,18,19,20,21,22,23]. The main problem with direct electroformation is that proteins and lipids are dried from organic solvent into a thin film, a harsh process which prohibits functional incorporation of most membrane enzymes [24]. Detergent-mediated reconstitution is a milder method and has been applied to GUV reconstitution for ion channels solubilized in sugar-based detergents [20]; however, this method has not been demonstrated for large eukaryotic transporters such as SERCA. Peptide-mediated fusion is a complicated procedure that shows variable efficiency and leaves fusogenic peptides in excess [19].

We propose that a Ca^{2+} transport assay utilizing GUV will overcome limitations of luminal Ca^{2+} and Ca^{2+} precipitants observed for SERCA in SR and SUV. The large volume of GUV should allow extended Ca^{2+} transport activity without buildup of intravesicular Ca^{2+} at inhibiting concentrations (0.1–1.0 mM). Here we show that SERCA can be functionally reconstituted in GUV via charge-mediated fusion (Fig. 1), which is a quick and mild method with four simple steps: (1) GUV electroformation with a positively-charged lipid mix, (2) standard SUV reconstitution of negatively-charged SERCA, (3) charge-mediated fusion to form GUV-SERCA, and (4) Ca^{2+} transport assay by confocal microscopy. In particular, this method incorporates SERCA in GUV by fusing preformed lipid-only GUV containing positively-charged phospholipid 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine chloride salt (EDOPC) to SUV containing SERCA, a highly-negative protein, reconstituted in neutral phospholipids 1,2-dioleoyl-sn-glycero-3phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE).

2. Materials and methods

2.1 Materials

DOPC, DOPE, EDOPC, and SUV extruder (100-nm pore) were purchased from Avanti Lipids. TR-DHPE, FITC, and Fluo-5F (cell-impermeant form) were purchased from Molecular Probes. TG, ATP, C_{12}E_8 , Triton X-100, Reactive Red-120 agarose, ITO-coated glass slides (70–100 Ω/sq , 75mm $\times$ 25mm $\times$ 1.1mm), and other chemicals were purchased from Sigma-Aldrich. BioBeads SM-2 was purchased from BioRad. The BCA protein assay kit was purchased from Pierce Technology. SR vesicles enriched in SERCA were purified from rabbit muscle using differential centrifugation [9]. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

2.2 Preparation of GUV-SERCA

Lipid-only GUV were prepared in by the electroformation method in 100 mM sucrose, 1 mM MOPS-Tris pH 7.0 (i.e., no added salts) [17,24,25]. SERCA was purified using affinity chromatography [26]. SERCA was labeled with FITC using manufacturer's instructions. SERCA and FITC-SERCA were reconstituted in SUV using the detergent-dilution method

[27]. Charge-mediate fusion was initiated by adding lipid GUV and SUV-SERCA together, followed by 4 hr on a rotary wheel at 4 °C. GUV-SUV fusion efficiency was detected using confocal microscopy and FITC-SERCA [28,29]. Ca^{2+} transport by GUV-SERCA was detected using confocal microscopy and encapsulated Fluo-5F [30]. To prevent GUV disruption by osmotic shock upon addition of salts and substrates in the Ca^{2+} transport assay solution [31], GUV-SERCA were transiently permeabilized with Triton X-100 at 200 μM (2.5-fold lower than CMC), and then GUV were re-sealed using Bio-Bead adsorption of Triton X-100. TG inhibition of Ca^{2+} transport by GUV-SERCA was assayed across the concentration range of 0–116 nM. The TG concentration required for 50% inhibition of SERCA activity (IC_{50}) was reported as mean $\pm$ standard error. The Supplemental Material contains additional details on final Methods (Sections S1–S8 on pages S2–S4) and protocol development (Figs. S1–S7 on pages S5–S11).

3. Results

3.1 Preparation of lipid-only GUV

The first step for GUV-SERCA reconstitution was to prepare positively-charged, lipid-only GUV (Fig. 1). GUV are prepared by methods such as electroformation, dehydration, and spontaneous swelling. We chose electroformation due to its ease, speed, yield, and reproducibility [19]. Cationic lipid GUV have been prepared by electroformation using neutral DOPC (net charge = 0) as the lipid base mixed with small amounts of positively-charged lipids (net-charge +1); for example, DOPC-GUV with 0–10% (wt/wt) of EDOPC, didodecyldimethylammonium (DDAB), or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). We previously used DOPC (base neutral lipid) and EDOPC (minor cationic lipid) to control membrane surface charge for functional co-reconstitution of SERCA and the cardiac regulatory subunit phospholamban in SUV, finding that SERCA activity is almost completely preserved ($>90\% V_{\text{max}}$) in SUV containing 5% or 10% EDOPC [32]. Cationic GUV composed of 85% DOPC and 15% EDOPC have been successfully grown by electroformation [25]. On the other hand, cationic GUV composed of 95% DOPC and 5% DDAB led to frequent adhesion of neighboring vesicles through shared membranes [33], while GUV composed of 95% DOPC + 5% DOTAP adhered strongly to ITO coverslips [25]. Thus, we chose EDOPC as the cationic lipid for GUV formation due to preservation of SERCA activity in SUV and to efficient formation of well-behaved GUV.

The concentration of EDOPC required for charge-mediated fusion with SUV is unknown, as is the upper limit of EDOPC, monovalent cation, and divalent cation allowed for GUV formation. First, the amount of EDOPC was systematically varied (0, 5, 10, 15, or 20%) and the efficiency of GUV electroformation was monitored using confocal microscopy. The neutral lipid dihexadecanoyl-glycero-3phosphoethanolamine (DHPE) labeled with the fluorescent dye Texas Red (TR) was added at trace amount (0.1%) to detect GUV formation (Fig. 2, 3, S2–S7). Here we observed that DOPC with trace TR-DHPE formed GUV efficiently using the electroformation method (lipid-coated ITO microscope slide subjected to alternating current for 3 hr), in agreement with previous reports [25]. The diameter of DOPC GUV ranged up to 100 μm , but typically we observed formation of GUV with diameter of 5–50 μm , which are suitable size for confocal microscopy analysis. Similar-

sized GUV were formed using 0, 5, 10, and 15% EDOPC. Other smaller vesicles were also formed by electroformation (diameter = 0.5–2.5 μm), but these ‘smaller’ GUV were not analyzed, since the size of these vesicles are only slightly above the resolution limit of standard confocal microscopy. Very few GUV were observed using electroformation of 20% EDOPC and 80% DOPC, and these GUV showed high tendency for GUV–GUV adherence (Fig. S2). Electroformation of GUV was greatly inhibited by 5 mM KCl or by 1 MgCl_2 (Fig. S3, S4). GUV with up to 15% EDOPC are stable in solution and exclude extravesicular Fluo-5F for 60 min without passive leakage (Fig. S5). Thus, GUV with 5, 10, and 15% EDOPC (cationic ‘helper’ lipid) were tested for fusion efficiency with negatively-charged SUV-SERCA proteoliposomes.

3.2 Preparation of SUV-SERCA

The second step to prepare GUV-SERCA (Fig. 1) is to reconstitute purified SERCA in SUV. We chose SERCA1a, the prototypical ion-motive ATPase protein, due to its well-established purification procedure from rabbit muscle, which gives high activity, high stability, and high yield [26]. We used a well-characterized procedure, detergent-dilution reconstitution, which uses many-fold dilution of the detergent below the detergent critical micelle concentration (CMC) in the presence of sufficient lipid to form unilamellar vesicles (reviewed by Rigaud, Pitard, and Lévy [27]). This method is rapid and suitable for membrane proteins purified in detergent solution and supplemented with lipid for storage (e.g., SERCA). Most importantly, detergent-dilution reconstitution yields highly-active SERCA in mostly-sealed SUV with SERCA in predominantly right-side-out orientation [34,35]. SUV reconstitution was performed using a mix of two neutrally-charged lipids (80% DOPC + 20% DOPC) that we have shown to maximize SERCA activity [36,37]. In general, reconstitution of SUV proteoliposomes requires a minimum of 100 lipids per protein [38]. Purified SERCA contains ≈ 8 co-purifying lipids (mol/mol) [38], so we typically supplemented purified SERCA with 50 lipids (mol/mol) for storage, and then added an additional 50 lipids (mol/mol) for during detergent-dilution reconstitution, giving a total of ≈ 110 lipid per SERCA in SUV-SERCA. The lipid/protein ratio of 110 was chosen because this condition provides efficient formation of SUV-SERCA with diameter $\sim 0.10\text{--}20\text{ }\mu\text{m}$ [39] and because SUV with this diameter range are fusogenic [40]. The negative charge of SUV-SERCA arises from rabbit SERCA1a, which is a 991-residue protein with a calculated molecular charge = -21 (Genbank sequence EU130921.1) and a measured isoelectric point $\text{pI} = 5.0$ [41]. FITC-SERCA was used to optimize GUV–SUV fusion as a function of EDOPC concentration (Fig. 2), while unlabeled SERCA was used for functional assays of Ca^{2+} transport and TG inhibition (Fig. 3).

3.3 Electrostatic fusion of lipid GUV and SUV-SERCA

The initiating step for bilayer–bilayer fusion is physical interaction and adhesion, which is enhanced by complementary membrane surface charge and curvature strain [31,42]. To evaluate the effect of membrane surface charge on GUV–SUV fusion (Step 3 in Fig. 1), GUV samples were prepared with varied content of EDOPC (0–20%). As shown in Fig. 2, fusion did not occur until the concentration of EDOPC in GUV reached 15%, as visualized by co-localization of FITC-labeled SERCA and TR-DHPE in GUV. GUV–SUV fusion was never observed with EDOPC 10 %, as assessed by many experiments and microscopy

slides analyzed through the entire project. Quantitative analysis of FITC-SERCA fluorescence in GUV demonstrated that increasing EDOPC from 15 to 20% increased GUV–SUV fusion by an additional $68 \pm 27\%$ (Fig. 2); however, 20% EDOPC severely inhibited lipid GUV formation (Fig. S2). At 15% EDOPC, the post-fusion distribution of FITC-SERCA was similar around the circumference of individual GUV (Fig. 2). The lipid/protein ratio of 110 was chosen because this condition provides efficient formation of SUV-SERCA with diameter $\sim 0.10\text{--}20\text{ }\mu\text{m}$ [39] and because SUV in this range of small diameter are fusogenic [40]. Since SERCA in SUV are mostly right-side-out [39], SERCA in GUV should maintain the correct orientation for functional assays due to the well-established directional fusion of membrane bilayers which does not induce protein flip-flop [19]. We previously showed that 10% EDOPC has insignificant effect on the rate of ATP hydrolysis by SERCA in SUV, although 20% EDOPC inhibited V_{max} by 10% [32]. Thus, 15% EDOPC was the concentration chosen for GUV-SERCA formation, due to the optimal balance of lipid GUV production, GUV–SUV fusion efficiency, and SERCA activity preservation.

3.4 Fluorescence microscopy assay of Ca^{2+} transport by GUV-SERCA

Ca^{2+} transport by GUV-SERCA was monitored using the fluorescence intensity of the Ca^{2+} indicator Fluo-5F encapsulated inside GUV. Fluo-5F ($20\text{ }\mu\text{M}$) was added during charge-mediated fusion. Following GUV–SUV fusion, 100 mM KCl and 1 mM MgCl_2 were added to GUV-SERCA to provide the physiological cations required for SERCA activity [26]. To prevent osmotic shock and disruption by addition of 100 mM KCl (Fig. S3, S6), GUV intended for Ca^{2+} transport assay were first permeabilized transiently with $200\text{ }\mu\text{M}$ Triton X-100. This small amount of Triton X-100 ($200\text{ }\mu\text{M}$), which is 2.5-fold below critical micellar concentration [43], does not solubilize GUV, nor does it allow Fluo-5F (molecular weight = 1101 Da) to pass through the GUV membrane bilayer (Figures S4–S7). After Triton addition, 100 mM KCl and 1 mM MgCl_2 were added sequentially to GUV, providing concentrations of K^+ and Mg^{2+} that give optimal SERCA activity [13]. Following ion equilibration, Triton X-100 and extravesicular Fluo-5F were removed by Bio-Bead adsorption, resulting in sealed GUV that maintained encapsulated Fluo-5F for 60 min.

Ca^{2+} transport assays were initiated by the addition of SERCA substrates at saturating concentration ($50\text{ }\mu\text{M Ca}^{2+}$ and 1 mM ATP) in order to monitor V_{max} activity. Fluo-5F fluorescence intensity shows a wide pseudo-linear response upon Ca^{2+} binding (increasing fluorescence), so radius-corrected fluorescence intensity was interpreted directly as SERCA-mediated Ca^{2+} transport [30]. In the absence of ATP, the fluorescence intensity of Fluo-5F in the interior of single GUV-SERCA remained at background level, demonstrating the lack of passive Ca^{2+} accumulation (Fig. 3A). Addition of ATP resulted in a 20-fold increase in Fluo-5F fluorescence intensity inside GUV, demonstrating ATP-driven Ca^{2+} transport by SERCA in GUV. Fluo-5F fluorescence was observed throughout the GUV interior, demonstrating that the intravesicular volume of GUV-SERCA is not restricted by interior lipid sheets, i.e., GUV-SERCA are unilamellar (Fig. 3A). Within single GUV, ATP-dependent Ca^{2+} transport continued to increase for 30 min, indicating that assays conducted for 10 min remain in the non-saturated phase of transport when conducted at $37\text{ }^\circ\text{C}$ in the presence of saturating concentrations of Ca^{2+} , ATP, and Mg^{2+} (Fig. 3). Ca^{2+} transport detection was compared for Fluo-5F versus Fluo-5N; we found that Fluo-5F gave higher

signal and higher signal-to-background than Fluo-5N, so Fluo-5F was chosen for transport assays. The stability and permeability of GUV were assayed by adding Fluo-5F to the outside of vesicles, which demonstrated the inability of extravesicular Fluo-5F to enter GUV by passive influx for 30–60 min (Fig. S5). Thus, GUV-SERCA are tightly sealed and functionally active.

3.5 Thapsigargin inhibition of GUV-SERCA

The optimized GUV-SERCA preparation was used to examine the inhibitory effect of TG on Ca^{2+} transport (Fig. 3). TG is a potent, plant-derived SERCA inhibitor which is currently in clinical trials for prostate cancer as the prodrug mipsagargin [44]. With increasing TG concentration, Ca^{2+} transport by SERCA was decreased, with an $\text{IC}_{50} = 11 \pm 1.6$ nM (Fig. 3), a value similar to that determined in native SR vesicles using ATPase assay in the presence of Ca^{2+} precipitating anion or Ca^{2+} ionophore [9,45]. Our transport data in GUV reveal that SERCA is sensitive to its classical inhibitor TG in the absence of Ca^{2+} precipitating anion (Fig. 3). Thus, GUV is validated as an ideal system to screen small-molecule effectors of SERCA.

4. Discussion

New methods are needed for reconstitution of membrane transport enzymes in GUV. For example, our experiments demonstrated that the Ca^{2+} transport activity of SERCA is abolished upon GUV reconstitution using the tradition method of solvent dehydration and direct electroformation. In 2004, Girard *et al.* reported a novel procedure to form GUV-SERCA using partial SERCA dehydration and direct electroformation, including preservation of transport activity [17]; however, this method has not been utilized subsequently. Our new method uses a well-defined system of synthetic lipids to functionally reconstitute SERCA via a mild and easy procedure.

Charge-mediated fusion may be a widely-applicable method for GUV incorporation. Membrane bilayer fusion is a process that proceeds through proximity, physical contact, and an energetic barrier transition that is facilitated by headgroup composition and bilayer strain curvature [42]. We maximized GUV-SUV fusion and SERCA activity by optimizing lipid composition and solution conditions; in particular, (i) 15% EDOPC was used as a helper lipid to facilitate GUV-SUV fusion, (ii) an SUV prep with small diameter was used to enhance fusogenicity, and (iii) 200 μM Triton X-100 was used for transient permeabilization of GUV-SERCA to allow addition of physiological salts and substrates required for assay of Ca^{2+} transport V_{max} .

During the preparation of this manuscript, an article was published reporting the reconstitution of bacterial membrane transport proteins using charge-mediated fusion [46]. In this study, Biner *et al.* fused negatively-charged lipid GUV (neutral DOPC + anionic DOPG) with positively-charged proteoliposome SUV (neutral DOPC + cationic DOTAP) containing two membrane protein complexes from bacteria, with successful preservation of functional activity [46]. Here we fused positively-charged lipid GUV with negatively-charged SUV proteoliposomes using the 110 kDa Ca^{2+} -transport ATPase (SERCA) from rabbit muscle as the model protein. Thus, the variety of these two reports by Biner *et al.* [46]

and Bian *et al.* [this study] suggests that charge-mediated fusion will serve as a new and widely-applied method for reconstitution and functional assay of membrane transport proteins in GUV.

5. Summary

In this report, we describe successful reconstitution of SERCA in GUV using charge-mediated fusion. We optimized lipid composition, protein preparation, and solution conditions to maximize GUV-SUV fusion, SERCA content, and functional activity. Ca^{2+} transport by GUV-SERCA was retained for 60 min and the inhibitory effect of TG was quantitatively detected, demonstrating the utility of this model membrane system to measure steady-state V_{max} activity in the absence of Ca^{2+} precipitating anions. Our protocol for GUV-SERCA preparation is well-designed (e.g., simple, robust, and efficient) and provides a means to test cellular conditions in a well-defined system with selected molecular components (e.g., lipid composition, protein post-translational modifications, and endogenous signaling molecules). Further case studies and charge-labeling strategies are needed to demonstrate that electrostatic fusion is a general method for reconstituting membrane proteins in GUV, including multimeric enzymes and regulatory complexes. We conclude that GUV is a versatile *in vitro* system for high-throughput screening of SERCA effectors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was funded by a graduate fellowship from the China Scholarship Council to T.B., and research grants from the United States National Institutes of Health to C.X. (CA163864) and D.D.T. (GM27906). SERCA purification was performed at the Biophysical Technology Center, University of Minnesota Department of Biochemistry, Molecular Biology, and Biophysics (<https://cbs.umn.edu/btc>). Jesse McCaffrey assisted with SERCA purification, Bengt Svensson assisted with molecular modeling, and Octavian Cornea assisted with manuscript preparation. Dynamic light scattering was performed in the laboratory of Jayanth Panyam, Department Head of Pharmaceutics, University of Minnesota. Fluorescence microscopy was performed using the Nikon NiE C2 upright confocal imaging system at the University of Minnesota Imaging Centers (<http://uic.umn.edu>), directed by Mark Sanders.

Abbreviations

CMC	critical micelle concentration
C_{12}E_8	octaethylene glycol monododecyl ether
DOPC	1, 2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine
EDOPC	1, 2-dioleoyl-sn-glycero-3-ethylphosphocholine
EGTA	ethylene glycolbis-(2-aminoethylether)-N,N,N',N' tetra-acetic acid

FITC	fluorescein-5-isothiocyanate
GUV-SERCA	giant unilamellar vesicle containing reconstituted SERCA
ITO	indium-tin oxide, OG, octyl β -D-glucopyranoside
SERCA	sarco/endoplasmic reticulum calcium transport ATPase
SR	sarcoplasmic reticulum
SUV-SERCA	small unilamellar vesicle containing reconstituted SERCA
TG	thapsigargin
TR-DHPE	n-Texas Red-sulfonyl-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine
Triton X-100	t-octylphenoxypolyethoxyethanol
V_{max}	maximal enzyme velocity at saturating substrate concentration.

References

1. Toyoshima C, Cornelius F. New crystal structures of PII-type ATPases: excitement continues. *Curr. Opin. Struct. Biol.* 2013; 23:507–514. [PubMed: 23871101]
2. Møller JV, Olesen C, Winther AM, Nissen P. The sarcoplasmic Ca^{2+} -ATPase: design of a perfect chemi-osmotic pump. *Q. Rev. Biophys.* 2010; 43:501–566. [PubMed: 20809990]
3. Lipskaia L, Keuylian Z, Blirando K, Mougenot N, Jacquet A, Rouxel C, Sghairi H, Elaib Z, Blaise R, Adnot S, Hajjar RJ, Chemaly ER, Limon I, Bobe R. Expression of sarco (endo) plasmic reticulum calcium ATPase (SERCA) system in normal mouse cardiovascular tissues, heart failure and atherosclerosis. *Biochim. Biophys. Acta.* 2014; 1843:2705–2718. [PubMed: 25110346]
4. Roti G, Carlton A, Ross KN, Markstein M, Pajcini K, Su AH, Perrimon N, Pear WS, Kung AL, Blacklow SC, Aster JC, Stegmaier K. Complementary genomic screens identify SERCA as a therapeutic target in NOTCH1 mutated cancer. *Cancer Cell.* 2013; 23:390–405. [PubMed: 23434461]
5. Cornea RL, Gruber SJ, Lockamy EL, Muretta JM, Jin D, Chen J, Dahl R, Bartfai T, Zsebo KM, Gillispie GD, Thomas DD. High-throughput FRET assay yields allosteric SERCA activators. *J. Biomol. Screen.* 2013; 18:97–107. [PubMed: 22923787]
6. Gruber SJ, Cornea RL, Li J, Peterson KC, Schaaf TM, Gillispie GD, Dahl R, Zsebo KM, Robia SL, Thomas DD. Discovery of enzyme modulators via high-throughput time-resolved FRET in living cells. *J. Biomol. Screen.* 2014; 19:215–222. [PubMed: 24436077]
7. Kang S, Dahl R, Hsieh W, Shin A, Zsebo KM, Buettner C, Hajjar RJ, Lebeche D. Small molecular allosteric activator of the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) attenuates diabetes and metabolic disorders. *J. Biol. Chem.* 2016; 291:5185–5198. [PubMed: 26702054]
8. Dahl R. A new target for Parkinson's disease: Small molecule SERCA activator CDN1163 ameliorates dyskinesia in 6-OHDA-lesioned rats. *Bioorg. Med. Chem.* 2016
9. Bleeker NP, Cornea RL, Thomas DD, Xing C. A novel SERCA inhibitor demonstrates synergy with classic SERCA inhibitors and targets multidrug-resistant AML. *Mol. Pharm.* 2013; 10:4358–4366. [PubMed: 24079514]
10. David-Bosne S, Clausen MV, Poulsen H, Møller JV, Nissen P, le Maire M, et al. Reappraising the effects of artemisinin on the ATPase activity of PfATP6 and SERCA1a E255L expressed in *Xenopus laevis* oocytes. *Nat. Struct. Mol. Biol.* 2016; 23:1–2. [PubMed: 26733217]

11. Inesi G, de Meis L. Regulation of steady state filling in sarcoplasmic reticulum. Roles of back-inhibition, leakage, and slippage of the calcium pump. *J. Biol. Chem.* 1989; 264:5929–5936. [PubMed: 2522442]
12. Inesi G, Tadini-Buoninsegni F. $\text{Ca}^{2+}/\text{H}^{+}$ exchange, lumenal Ca^{2+} release and $\text{Ca}^{2+}/\text{ATP}$ coupling ratios in the sarcoplasmic reticulum ATPase. *J. Cell. Commun. Signal.* 2014; 8:5–11. [PubMed: 24302441]
13. Autry JM, Jones LR. Functional co-expression of the canine cardiac Ca^{2+} pump and phospholamban in *Spodoptera frugiperda* (Sf21) cells reveals new insights on ATPase regulation. *J. Biol. Chem.* 1997; 272:15872–15880. [PubMed: 9188486]
14. Karon BS, Nissen ER, Voss J, Thomas DD. A continuous spectrophotometric assay for simultaneous measurement of calcium uptake and ATP hydrolysis in sarcoplasmic reticulum. *Anal. Biochem.* 1995; 227:328–333. [PubMed: 7573954]
15. Sehgal P, Olesen C, Møller JV. Ca^{2+} binding and transport studied with $\text{Ca}^{2+}/\text{EGTA}$ buffers and $(45)\text{Ca}^{2+}$. *Methods Mol. Biol.* 2016; 1377:261–266. [PubMed: 26695038]
16. Sehgal P, Olesen C, Møller JV. Coupling ratio for Ca^{2+} transport by calcium oxalate precipitation. *Methods Mol. Biol.* 2016; 1377:157–160. [PubMed: 26695030]
17. Girard P, Pecreaux J, Lenoir G, Falson P, Rigaud JL, Bassereau P. A new method for the reconstitution of membrane proteins into giant unilamellar vesicles. *Biophys. J.* 2004; 87:419–429. [PubMed: 15240476]
18. Dezi M, Di Cicco A, Bassereau P, Lévy D. Detergent-mediated incorporation of transmembrane proteins in giant unilamellar vesicles with controlled physiological contents. *Proc. Nat. Acad. Sci. U.S.A.* 2013; 110:7276–7281.
19. Kahya N, Pecheur EI, de Boeij WP, Wiersma DA, Hoekstra D. Reconstitution of membrane proteins into giant unilamellar vesicles via peptide-induced fusion. *Biophys. J.* 2001; 81:1464–1474. [PubMed: 11509360]
20. Kreir M, Farre C, Beckler M, George M, Fertig N. Rapid screening of membrane protein activity: electrophysiological analysis of OmpF reconstituted in proteoliposomes. *Lab Chip.* 2008; 8:587–595. [PubMed: 18369514]
21. Yanagisawa M, Iwamoto M, Kato A, Yoshikawa K, Oiki S. Oriented reconstitution of a membrane protein in a giant unilamellar vesicle: experimental verification with the potassium channel KcsA. *J. Am. Chem. Soc.* 2011; 133:11774–11779. [PubMed: 21702488]
22. Horger KS, Liu H, Rao DK, Shukla S, Sept D, Ambudkar SV, Mayer M. Hydrogel-assisted functional reconstitution of human P-glycoprotein (ABCB1) in giant liposomes. *Biochim. Biophys. Acta.* 2015; 1848:643–653. [PubMed: 25450342]
23. Smeazzetto S, Tadini-Buoninsegni F, Thiel G, Berti D, Montis C. Phospholamban spontaneously reconstitutes into giant unilamellar vesicles where it generates a cation selective channel. *Phys. Chem. Chem. Phys.* 2016; 18:1629–1636. [PubMed: 26673394]
24. Meleard P, Bagatolli LA, Pott T. Giant unilamellar vesicle electroformation from lipid mixtures to native membranes under physiological conditions. *Methods Enzymol.* 2009; 465:161–176. [PubMed: 19913167]
25. Herold C, Chwastek G, Schwille P, Petrov EP. Efficient electroformation of supergiant unilamellar vesicles containing cationic lipids on ITO-coated electrodes. *Langmuir.* 2012; 28:5518–5521. [PubMed: 22424289]
26. Reddy LG, Jones LR, Cala SE, O'Brian JJ, Tatulian SA, Stokes DL. Functional reconstitution of recombinant phospholamban with rabbit skeletal Ca^{2+} -ATPase. *J. Biol. Chem.* 1995; 270:9390–9397. [PubMed: 7721863]
27. Rigaud JL, Pitard B, Lévy D. Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins. *Biochim. Biophys. Acta.* 1995; 1231:223–246. [PubMed: 7578213]
28. Autry JM, Rubin JE, Pietrini SD, Winters DL, Robia SL, Thomas DD. Oligomeric interactions of sarcolipin and the Ca^{2+} -ATPase. *J. Biol. Chem.* 2011; 286:31697–31706. [PubMed: 21737843]
29. Autry JM, Rubin JE, Svensson B, Li J, Thomas DD. Nucleotide activation of the Ca^{2+} -ATPase. *J. Biol. Chem.* 2012; 287:39070–39082. [PubMed: 22977248]

30. Eaddy AC, Schnellmann RG. Visualization and quantification of endoplasmic reticulum Ca²⁺ in renal cells using confocal microscopy and Fluo5F. *Biochem. Biophys. Res. Comm.* 2011; 404:424–427. [PubMed: 21130732]
31. Shirt-Ediss B, Sole RV, Ruiz-Mirazo K. Emergent chemical behavior in variable-volume protocells. *Life.* 2015; 5:181–211. [PubMed: 25590570]
32. Li J, James ZM, Dong X, Karim CB, Thomas DD. Structural and functional dynamics of an integral membrane protein complex modulated by lipid headgroup charge. *J. Mol. Biol.* 2012; 418:379–389. [PubMed: 22381409]
33. Bucher P, Fischer A, Luisi PL, Oberholzer T, Walde P. Giant vesicles as biochemical compartments: the use of microinjection techniques. *Langmuir.* 1988; 14:2712–2721.
34. Van Winkle WB, Pitts BJ, Entman ML. Rapid purification of canine cardiac sarcoplasmic reticulum Ca²⁺-ATPase. *J. Biol. Chem.* 1978; 253:8671–8673. [PubMed: 152759]
35. le Maire M, Møller JV, Champeil P. Binding of a nonionic detergent to membranes: flip-flop rate and location on the bilayer. *Biochemistry.* 1987; 26:4803–4810. [PubMed: 3663628]
36. Cornea RL, Thomas DD. Effects of membrane thickness on the molecular dynamics and enzymatic activity of reconstituted Ca-ATPase. *Biochemistry.* 1994; 33:2912–2920. [PubMed: 8130205]
37. Reddy LG, Autry JM, Jones LR, Thomas DD. Co-reconstitution of phospholamban mutants with the Ca-ATPase reveals dependence of inhibitory function on phospholamban structure. *J. Biol. Chem.* 1999; 274:7649–7655. [PubMed: 10075652]
38. Lacapere JJ, Stokes DL, Olofsson A, Rigaud JL. Two-dimensional crystallization of Ca-ATPase by detergent removal. *Biophys. J.* 1998; 75:1319–1329. [PubMed: 9726933]
39. Lévy D, Gulik A, Bluzat A, Rigaud JL. Reconstitution of the sarcoplasmic reticulum Ca(2+)-ATPase: mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. *Biochim. Biophys. Acta.* 1992; 1107:283–298. [PubMed: 1387003]
40. Bentz J, Duzgunes N. Fusogenic capacities of divalent cations and effect of liposome size. *Biochemistry.* 1985; 24:5436–5443. [PubMed: 4074706]
41. le Maire M, Jorgensen KE, Roigaard-Petersen H, Møller JV. Properties of deoxycholate solubilized sarcoplasmic reticulum Ca²⁺-ATPase. *Biochemistry.* 1976; 15:5805–5812. [PubMed: 137739]
42. Fuhrmans M, Marelli G, Smirnova YG, Muller M. Mechanics of membrane fusion/pore formation. *Chem. Phys. Lipids.* 2015; 185:109–128. [PubMed: 25087882]
43. Li N, Luo H, Liu S. A new method for the determination of the critical micelle concentration of Triton X-100 in the absence and presence of beta-cyclodextrin by resonance Rayleigh scattering technology. *Spectrochim. Acta Mol. Biomol. Spectrosc.* 2004; 60:1811–1815.
44. Doan NT, Paulsen ES, Sehgal P, Møller JV, Nissen P, Denmeade SR, Isaacs JT, Dionne CA, Christensen SB. Targeting thapsigargin towards tumors. *Steroids.* 2015; 97:2–7. [PubMed: 25065587]
45. Yu M, Zhong L, Rishi AK, Khadeer M, Inesi G, Hussain A. Specific substitutions at amino acid 256 of the sarcoplasmic/endoplasmic reticulum Ca²⁺ transport ATPase mediate resistance to thapsigargin in thapsigargin-resistant hamster cells. *J. Biol. Chem.* 1998; 273:3542–3546. [PubMed: 9452480]
46. Biner O, Schick T, Muller Y, von Ballmoos C. Delivery of membrane proteins into small and giant unilamellar vesicles by charge-mediated fusion. *FEBS Lett.* 2016; 590:2051–2062. [PubMed: 27264202]
47. Toyoshima C, Nomura H. Structural changes in the calcium pump accompanying the dissociation of calcium. *Nature.* 2002; 418:605–611. [PubMed: 12167852]

Fig. 1. Schematic formation and functional assay of GUV-SERCA

Lipid GUV and SUV-reconstituted SERCA were mixed to induce GUV-SERCA formation (steps 1–3). Ca^{2+} transport and TG inhibition were detected via fluorescence imaging of Fluo-5F encapsulated in GUV-SERCA (step 4). The scale of components is not proportional to actual sizes.

Fig. 2. Effect of EDOPC concentration on charge-mediated fusion of lipid GUV and SUV-SERCA

SUV containing FITC-SERCA were mixed with lipid GUV composed of a mixture of DOPC and EDOPC. (*Top*) Confocal microscopy images identify GUV-SERCA formation. (*Bottom*) The amount of SERCA incorporated in GUV was quantified by image analysis of FITC fluorescence line density. Relative GUV-SUV fusion efficiency was normalized to 15% EDOPC (fusion $\equiv 1.0$) and reported as mean $\pm$ standard error (N=14–23).

Fig. 3. Dose-dependent response for thapsigargin inhibition of calcium transport in GUV-SERCA

GUV with unlabeled SERCA, encapsulated Fluo-5F, and a lipid mixture of 15% EDOPC, 85% DOPC, and 0.1% TR-DHPE were assayed for Ca^{2+} transport and TG inhibition. (*Top*) Quantitative microscopy of Fluo-5F fluorescence. (*Bottom*) Concentration dependence of TG inhibition. SERCA transport activity was normalized to 0 nM TG ($V_{\text{max}} \equiv 1.0$) and reported as mean $\pm$ standard error ($N=7-17$). The TG-dependence of Ca^{2+} transport

inhibition (IC_{50}) was fit to the Hill equation, assuming one TG binding site per SERCA [45,47] (Fig. S1).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript