Mechanisms for cardiac SERCA activation by its substrate and a synthetic allosteric modulator using fluorescence lifetime imaging

Jaroslava Šeflová,‡ Carlos Cruz-Cortés,§ Guadalupe Guerrero-Serna,§ Seth L. Robia,‡ and L. Michel Espinoza-Fonseca§,*

‡Center for Arrhythmia Research, Department of Internal Medicine, Division of Cardiovascular Medicine, University of Michigan, Ann Arbor, MI 48109, USA; §Department of Cell and Molecular Physiology, Loyola University Chicago, Maywood, IL 60153, USA.

Abstract: The discovery of allosteric modulators is an emerging paradigm in drug discovery, and signal transduction is a subtle and dynamic process that is challenging to characterize. We developed a time-correlated single photon counting (TCSPC) imaging approach to investigate the structural mechanisms for small-molecule activation of the cardiac sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA2a), a pharmacologically important pump that transports Ca$^{2+}$ at the expense of ATP hydrolysis. We first tested whether the dissociation of SERCA2a from its regulatory protein phospholamban (PLN) is required for small-molecule activation. We found that CDN1163, a validated SERCA2a activator, does not have significant effects on the stability of the SERCA2a–PLN complex. TCSPC imaging experiments using the non-hydrolyzable ATP analog AMP-PCP showed ATP is an allosteric modulator of SERCA2a, increasing the fraction of catalytically competent structures at physiologically relevant Ca$^{2+}$ concentrations. Unlike ATP, CDN1163 alone has no significant effects on the Ca$^{2+}$-dependent shifts in the structural populations of SERCA2a, and it does not increase the pump’s affinity for Ca$^{2+}$ ions. However, we found that CDN1163 enhances the ATP-mediated modulatory effects to increase the population of catalytically competent SERCA2a structures. Importantly, this structural shift occurs within the physiological window of Ca$^{2+}$ concentrations at which SERCA2a operates. We demonstrated that ATP is both a substrate and modulator of SERCA2a and showed that CDN1163 and ATP act synergistically to populate SERCA2a structures that are primed for phosphorylation. This study provides novel insights into the structural mechanisms for SERCA2a activation by its substrate and a synthetic allosteric modulator.

Significance Statement: Allosteric modulation is a growing concept in drug discovery, yet unraveling the structural mechanisms that underlie this phenomenon continues to pose a challenge. We developed a time-correlated single photon counting (TCSPC) imaging approach to investigate the structural mechanisms for allosteric activation of the cardiac calcium pump SERCA2a, a pharmacological target for the treatment of heart failure. We established that ATP, the molecule that fuels SERCA2a, is both a substrate and modulator of the pump and showed that a synthetic allosteric activator enhances the ATP-mediated modulatory effects to populate SERCA2a structures that are ready for SERCA2a phosphorylation. This study shows the synergy between SERCA2a’s substrate and a synthetic allosteric modulator to activate a clinically important target in the heart.
Introduction

The concept of allostery for drug discovery is a growing paradigm in medicinal chemistry.\(^1\) In this paradigm, allosteric drugs modulate the activity through the propagation of allosteric signaling, producing either inhibition or activation of the target. Understanding the structural mechanisms for small-molecule modulation is an essential prerequisite for allosteric drug discovery,\(^3\) but signal transduction is a dynamic process involving subtle structural changes\(^4\) that are challenging to characterize using traditional experimental techniques.\(^6\) This motivates the development of novel approaches to determine the structural mechanisms underlying small-molecule allosteric modulation of druggable proteins.

The cardiac calcium pump (Sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, SERCA2a) plays an essential role in normal cardiac function, clearing cytosolic Ca\(^{2+}\) needed to relax muscle cells in each heartbeat (diastole).\(^7\) SERCA pumps two Ca\(^{2+}\) ions into the SR lumen using energy derived from the hydrolysis of ATP\(^8\) and is regulated by the 52-residue membrane protein phospholamban (PLN). PLN regulates SERCA2a by inhibiting its ability to transport Ca\(^{2+}\),\(^10\) and inhibition is relieved by phosphorylation of PLN.\(^12\) A key molecular dysfunction in patients with heart failure usually involves insufficient SERCA expression and impaired PLN phosphorylation, leading to SERCA2a inactivation and decreased Ca\(^{2+}\) transport in the cardiomyocyte. Reactivation of Ca\(^{2+}\) transport results in improved cardiac function in experimental models of heart failure,\(^17\)\(^-\)\(^21\) and SERCA2a is a well-validated target and its small-molecule activation is a promising approach for heart failure therapy.\(^22\)\(^-\)\(^23\)

SERCA’s structure, function, and regulation are the subjects of hundreds of papers (see recent reviews)\(^24\)\(^-\)\(^28\) yet there is not a single study addressing the mechanisms for the small-molecule activation of this pump. In addition, there are no crystal structures of SERCA bound to small-molecule activators, so the structural basis for small-molecule SERCA activation remains enigmatic. This has limited systematic, structure-based drug discovery and hit-to-lead optimization campaigns aimed at the discovery of new therapeutic products targeting this pharmacologically important pump. In this study, we introduce a time-correlated single photon counting (TCSPC) imaging method to investigate in unprecedented detail the mechanisms for small-molecule SERCA2a activation. This approach revealed novel features of small-molecule activation of SERCA2a, including the concept that SERCA2a activation by a small-molecule effector does not require dissociation of the SERCA2a–PLN complex, the notion that ATP is both a substrate and an effector of the pump and that stimulation of SERCA2a arises from the synergy between the activator and ATP to populate structures primed for SERCA2a phosphorylation. The result is a vivid visualization of the molecular mechanism for the small-molecule activation of a druggable target in the heart.

Materials and methods

*Corresponding Author
L. Michel Espinoza–Fonseca – Center for Arrhythmia Research, Department of Internal Medicine, Division of Cardiovascular Medicine, University of Michigan, Ann Arbor, MI 48109, USA; E-mail: lemef@umich.edu.
Chemicals. All chemicals used in this study were purchased at reagent quality (purity>95% by HPLC): CDN1163, N-(2-methylquinolin-8-yl)-4-propan-2-yloxybenzamide (Sigma, St. Louis, MO); istaroxime, (3E,5S,8R,9S,10R,13S,14S)-3-(2-aminoethoxyimino)-10,13-dimethyl-1,2,4,5,7,8,9,11,12,14,15,16-dodecacycloheptap[a]phenanthrene-6,17-dione (MedChemExpress LLC, Monmouth Junction, NJ); CP-154526, N-butyl-N-ethyl-2,5-dimethyl-7-(2,4,6-trimethylphenyl)pyrrolo[2,3-d]pyrimidine-4-amine (Sigma, St. Louis, MO), Ro 41-0960, (3,4-dihydroxy-5-nitrophenyl)-(2-fluorophenyl) methanone (Sigma, St. Louis, MO); AMP-PCP, β,γ-Methyleneadenosine-5′-triphosphate (Sigma, St. Louis, MO).

Isolation of enriched SERCA2a microsomes. Pig hearts were obtained after euthanasia and placed in a cardioplegic solution (280 mM glucose, 13.44 mM KCl, 12.6 mM NaHCO₃, and 34 mM mannitol). Left ventricles free walls were obtained, minced, and homogenized with a cold buffer that contained 9.1 mM NaHCO₃, 0.9 mM Na₂CO₃, and a cocktail of proteases inhibitors (Sigma, St. Louis, MO); the mixture was centrifuged at 6,500 g for 30 minutes at 4°C to remove debris. The supernatant was filtered, collected, and centrifuged at 14,000 g for 30 min at 4°C. The collected filtrate was centrifuged at 47,000 g for 60 min at 4°C. The pellet was resuspended in a solution containing 0.6 M KCl and 20 mM Tris (pH=6.8). The suspension was centrifuged at 120,000 g for 60 min at 4°C, and the pellet was resuspended in a solution containing 0.3 M sucrose, 5 mM MOPS, and protease inhibitors (pH=7.4). The protein concentration of the SR microsomal fraction was determined using the PierceTM Coomassie plus assay kit (Thermo-Fisher Scientific, Waltham, MA). The microsomal membranes were aliquoted, frozen in liquid nitrogen, and stored at -80°C. SERCA2a purification was performed as described by Sitsel et al.²⁹

Western Blot analysis. Samples were loaded into 4-20% Tris-Glycine polyacrylamide precast gels (ThermoFisher Scientific, Waltham, MA USA) and electrophoresis was carried out. The SDS-PAGE resolved proteins were transferred to iBlot stacks with regular PVDF membranes using the iBlot 2 Dry Blotting System (ThermoFisher Scientific, Waltham, MA USA). Nonspecific binding sites were blocked with 5% nonfat dry milk in PBS-T (in mmol/L, 3 KH₂PO₄, 10 Na₂HPO₄, 150 NaCl, 0.15% Tween 20, pH 7.2-7.4) for 30 min at room temperature. Membranes were then incubated with specific primary antibodies for SERCA2a (1:500; ThermoFischer) and PLN (1:5000; Badrilla, UK), diluted in 5% bovine serum albumin in PBS-T overnight at 4°C. After washing 3 times for 10 minutes, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, diluted in 5% bovine serum albumin in PBS-T. After washing 3 times for 10 minutes, protein-antibody reactions were detected using Pierce SuperSignal Chemiluminescent Substrates (ThermoFisher Scientific, Waltham, MA USA). We performed detection and quantification of protein bands with a Bio-Rad ChemiDoc system and Image Lab software 5 (Bio-Rad, Hercules, CA USA).

SERCA ATPase activity assays. We performed SERCA2a activity assays using an enzyme-coupled NADH-linked ATPase assay described previously.³⁰ Briefly, we measured the activity of Ca²⁺ ATPase in µmol min⁻¹ mg⁻¹ from the decrease in absorbance of NADH at 340 nm at 25°C in a 96-well format using a Synergy H1 (BioTek, Winooski, VT) microplate reader. Each well contained a 200 µl final volume of assay buffer containing SERCA buffer (50 mM MOPS, 100 mM KCl, 5 mM MgCl₂, and 1 mM EGTA, pH=7), 5U lactate dehydrogenase, 5U pyruvate dehydrogenase, 1 mM
phosphoenolpyruvate, 5 mM ATP, 0.2 mM NADH, 2 µg of microsomal suspension, 2 µM of Ca\textsuperscript{2+} ionophore A23187, and eight free Ca\textsuperscript{2+} concentrations. Each concentration of the compounds tested here was calculated to a final volume of 200 µl. We incubated the small molecules for 30 minutes at 25°C with the reaction mixture. Concentration-response curves for each compound were constructed with the data from [Ca\textsuperscript{2+}]-dependent SERCA activity curves performed at compound concentrations of 0.1-100 µM. The final free Ca\textsuperscript{2+} concentrations were calculated using MaxChelator, and were achieved by using twelve individual stock CaCl\textsubscript{2} solutions. Each plate included untreated and thapsigargin-treated microsomes as controls. To account for biological variability, we use microsomal fractions obtained from three pig hearts. In all cases, the maximal activity values (V\textsubscript{max}) were normalized relative to the compound-free samples.

**HEK-293T cells culture.** The human two-color SERCA2a construct was produced as described previously. The mCerulean at the N-terminus of human SERCA2a was replaced by mCyRFP\textsuperscript{133} and YFP was replaced by mMaroon\textsuperscript{134} at the position before residue number 509 within the N-domain of SERCA2a (Fig. 1A). For microsomes containing SERCA2a and PLN, the mCyRFP\textsuperscript{1} and mMaroon were fused to the N-termini of SERCA2a and PLN, respectively. The mCyRFP\textsuperscript{1}-mMaroon\textsuperscript{1} construct has a Förster distance of 63.34 Å.\textsuperscript{33, 36} The final constructs were verified by full-length sequencing (ACGT, USA). HEK 293T cells (CRL-3216, ATCC) were seeded into a 150 mm cell culture dish 72 hours before transient transfection and cultured in DMEM (Corning, USA) supplemented with 10% FBS in 5% CO\textsubscript{2} incubator at 37°C. When cells reached 70-80% confluence, the cell culture media was replaced by fresh media, and cells were transfected with two-color SERCA construct\textsuperscript{32, 36} using Lipofectamine 3000 kit (Invitrogen, Life Technologies). Cells were harvested 48 hours post-transfection and microsomal membranes were prepared on the day of the harvest.

**Microsomal membranes preparation from transfected HEK-293T cells.** The microsomal membranes from HEK-293T were prepared according to the protocol published previously.\textsuperscript{32, 37} Specifically, each 150 mm cell culture dish was scraped into 12 ml of homogenization buffer (0.5 mM MgCl\textsubscript{2}, 10 mM Tris-HCl; pH 7.5) supplemented with UltraCruz Protease Inhibitor Cocktail Tablet without EDTA (Santa Cruz Biotechnology). Cells were pelleted using centrifugation of 1,000 g for 10 minutes at 4°C and subsequently dissolved in 5 ml of fresh homogenization buffer. The cell suspension was homogenized with 20 strokes of Potter-Elvehjem glass homogenizer, and 5 ml of sucrose buffer (100 mM MOPS, 500 mM sucrose; pH=7.0 with protease inhibitors). The crude homogenate was passed 10 times through a 27-gauge needle. Subsequently, the homogenate was centrifuged at 1,000 g for 10 minutes at 4°C to remove unbroken cells, mitochondria, and cellular debris. The supernatant was subjected to ultracentrifugation at 126,000 g for 30 minutes at 4°C, and the pellet was dissolved in a 1:1 mixture of homogenization and sucrose buffers. The total protein concentration was determined using BCA assay (Pierce BCA Protein assay, ThermoFisher Scientific, Rockford, IL). The microsomal fractions were separated into single-use aliquots containing 50 μl of microsomal membranes at a concentration of 3-4.5 mg/ml. We performed four independent transfections to account for biological variability as part of our experimental design, so each experiment represents a biological replicate.

**Microsomal membranes preparation from HEK-293T cells infected with adenovirus.** The 90% confluent cells were infected with human type 5 (dE/E3) adenovirus encoding two
color SERCA (Vector Biolabs, USA). The multiplicity of infection was 35. This value was experimentally determined using a small-scale infection. Infected cells were harvested 48 hours post-transfection and microsomal membranes were prepared using the same methodology as described for transiently transfected HEK-293T cells.

PLN-CDN1163 competition experiments. The SERCA2a competition experiment monitored FRET changes between mCyRFP1-SERCA2a and mMaroon1-PLN microsomes. We used a physiologically relevant SERCA-to-PLN DNA ratio of 1:5 and tested the effects of CDN1163 at increasing concentrations of the compound (0.1–50 μM). The donor alone lifetime was shortened to 2.63 ns due to FRET. This corresponds to average FRET efficiency of 26% using the following equation $FRET\ Efficiency\ (%) = 100 \times \left(1 - \frac{\tau_{DA}}{\tau_D}\right)$, where $\tau_D$ is the lifetime of donor alone and $\tau_{DA}$ is the lifetime of the donor in the presence of acceptor.

Time-correlated single-photon counting imaging of SERCA2a. Aliquots of microsomes were thawed on ice, mixed with the DMSO vehicle (0.01%) or 10 μM CDN1163 (>98% purity by HPLC; Sigma-Aldrich), and incubated for 30 minutes at room temperature. Subsequently, the microsomes were mixed with a buffer composed of 120 mM potassium aspartate, 15 mM KCl, 5 mM KH₂PO₄, 0.75 mM MgCl₂, 2% dextran, 20 mM HEPES, 2 mM EGTA, and CaCl₂ 1.7 mM; pH 7.2 with or without 500 μM AMP-PCP and imaged immediately after mixing. The free [Ca²⁺] concentrations were estimated to range from 0.001 to 100 μM. The final free Ca²⁺ concentrations were calculated using MaxChelator, and were achieved by using twelve individual stock CaCl₂ solutions. Time-correlated single-photon counting (TCSPC) experiments were performed as previously described. Donor (mCyRFP1) fluorescence in the membrane microsomes was excited by a supercontinuum laser beam (Fianium, Southampton, United Kingdom). The donor signal was acquired using an excitation filter 482/18 nm bandpass filter. Fluorescence emission was detected using a 525/50 nm emission bandpass filter. The focused laser was placed inside of microsomes drop, yielding a 100,000 photons/s count rate. Under those conditions, we observed less than 10% photobleaching during the 60 seconds of acquisition time. Fluorescence was detected through a 1.2 N.A. water-immersion objective with a PMA hybrid detector (PicoQuant, Germany) connected to a single photon-counting module (HydraHarp 300, PicoQuant, Germany) with a time channel width of 16 ps. The TCSPC imaging method has over 4 orders of magnitude difference between signal and noise (Fig. 1B), making it ideal for capturing the subtle structural changes associated with allosteric modulation.

Global analysis of the fluorescence lifetimes. Fluorescence decay histograms from 4 different sets of microsomes were analyzed in SymPhoTime 64 software (Picoquant, Germany) with the TCSPC global fitting tool. The donor alone (mCyRFP1-SERCA2a) showed slightly two-exponential decay with relative amplitude of the second component accounting for less than 8%, therefore the donor alone lifetime was estimated as a single exponential decay with a lifetime of 3.56 ns for all tested conditions. The donor in the presence of the acceptor (two-color SERCA2a or mMaroon1-PLN) was best fitted using a two-exponential decay, where the amplitude-weighted average lifetime ($\tau_{avg}$) was shorter than the lifetime of the donor alone that is consistent with FRET between donor and acceptor. We performed fitting with the increasing complexity of the used model (increasing number of exponential functions used in the fit) and we evaluated the
residual distribution of the performed fit and $\chi^2$. Therefore, the two-exponential model was selected as the correct model describing the decay of 2-color SERCA2a. Each of the fitted exponential models describes one component that is present in the overall decay and % content of this component. In addition, we performed two-exponential fitting of all experimental conditions and determined changes in both individual lifetimes. The values of these two individual lifetimes did not vary significantly within all tested groups which is suggestive of two structural species that change their relative % content (populations of fluorescent species). These changes in the populations are reflected as changes in the amplitude-weighted average lifetimes. We further performed a global fitting that allows to share selected lifetimes within all experimental groups which eliminates variability in the used mathematical model. We assume the presence of two populations of SERCA2a, open and closed, that are resolved by TCSPC, which agrees with our recent work.  

**Statistical analysis.** All results are presented as mean ± standard error of the mean (SEM). Significance was evaluated using the Mann–Whitney U test for paired experiments or a two-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test to analyze differences between the control and multiple treatments. We used 95% confidence intervals around the differences between the groups for the post-hoc test. Two-sided $p$ values were used, and $\alpha$-level <0.05 was considered significant.

**Results**

**ATPase assays validate the synthetic molecule CDN1163 as a SERCA2a activator**

There are only a handful of small molecules that have been reported to stimulate SERCA activity: CDN1163, an allosteric activator that stimulates the non-muscle SERCA isoform SERCA2b $^{40,41}$ istaroxime, a molecule that was proposed to stimulate SERCA2a and inhibits the Na$^+$/K$^+$-ATPase (NKA) $^{42}$ CP-154526, which was reported to increase the maximal activity of muscle SERCA isoforms $^{43}$ and Ro 41-0960, a small molecule that increases both the activity and Ca$^{2+}$ affinity of the SERCA2a pump $^{43}$ (Fig. 2A). The full activation profiles of these molecules have not yet been reported in the literature, therefore we first used ATPase activity assays to establish the concentration-response profiles of these compounds on SERCA2a. In all cases, the SERCA2a activity is reported as the change of $V_{\text{max}}$ relative to the activity of SERCA2a alone. When describing the data, we distinguish between ‘observed’ data (i.e., data points) and ‘estimated’ data (i.e., values derived from the fitted model data).

CDN1163 activates SERCA2a in a concentration-dependent manner with an EC$^{50}$ value of 2.3 µM, and a maximal increase in the relative $V_{\text{max}}$ activity of 11.8% estimated from the fitted curve (Fig. 2B). Notably, we found that the stimulatory effect of CDN1163 on SERCA2a remains constant at compound concentrations >10 µM (Fig. 2B). In our hands, istaroxime does not activate SERCA2a at any compound concentration (Fig. 2C). At high concentrations, istaroxime has a slight inhibitory effect on SERCA2a; however, this effect is not significant. We speculate that istaroxime does not activate SERCA2a in our assays, probably because of species-specific differences. $^{44}$ However, we note additional ATPase assays performed by us showed that istaroxime inhibits the cardiac isoform of the $\alpha_1$ isoform of pig Na$^+$/K$^+$-ATPase (NKA-$\alpha_1$) with an IC$^{50}$ = 0.47 µM; this IC$^{50}$ value is virtually identical to that previously reported using the dog NKA-$\alpha_1$ (IC$^{50}$ = 0.43 µM). $^{45}$ These findings suggest that, unlike CDN1163, istaroxime may be a species-specific activator of the SERCA2a pump. CP-154526 also activates SERCA2a
in the high nM to low µM range, with an observed maximal increase in $V_{\text{max}}$ of 28% at a compound concentration of 2.5 µM (Fig. 2D). However, this compound follows a bell-shaped dose-response curve, where increasing compound concentration leads to increased activity up to a point and further increases in compound concentration lead to decreasing or abolished activity. This effect is likely the result of CP-154526 aggregation, as suggested by atomistic molecular dynamics simulations. The ATPase activation assays showed that Ro 41-0960 does not stimulate the activity of SERCA2a in the concentration range tested here (0.1-100 µM, Fig. 2E). Instead, Ro 41-0960 has an inhibitory activity at compound concentrations ≥25 µM, with an observed decrease in $V_{\text{max}}$ by ~30% at a compound concentration of 100 µM (Fig. 2E). In summary, only CDN1163 activates SERCA2a in a concentration-response manner, hence we used this effector as a molecular probe to study the mechanisms for SERCA2a activation.

**CDN1163 does not dissociate the SERCA–PLN complex**

A conventional concept in the field is that dissociation of the endogenous regulatory SERCA–PLN complex is a requirement for small-molecule activation of the calcium pump. However, Western blot analysis showed that SERCA2a purification removes most PLN that is present in the cardiac SR microsomal fraction (Fig. 3A). In this SERCA2a preparations devoid of PLN, we have measured a maximal ATPase activity that falls within the activity range usually obtained using SR microsomes, e.g., 0.46 µmol min$^{-1}$ mg$^{-1}$ for the purified SERCA (Fig 3B) vs a $V_{\text{max}}$ of 0.4-0.6 µmol min$^{-1}$ mg$^{-1}$ typically observed in SR microsomal preparations in our lab. Therefore, the removal of PLN does not increase SERCA2a’s turnover rate. Based on this evidence, we propose the hypothesis that CDN1163 does not activate SERCA2a by dissociating PLN from the SERCA2a–PLN complex.

We tested this hypothesis using fluorescently labeled SERCA2a and PLN. Here, binding of PLN to SERCA2a produces a FRET signal (measured as the % of FRET efficiency); dissociation of the complex by CDN1163 is shown by the decrease of the FRET signal between SERCA2a and PLN (dissociation model) whereas no changes in the SERCA2a–PLN FRET efficiency suggest that CDN1163 binds to the regulatory complex (subunit model) (Fig. 4A). Activation of SERCA2a requires both Ca$^{2+}$ and ATP, so we performed these studies at low (0.01 µM) and high (10 µM) free Ca$^{2+}$ concentrations, as well as in the presence and absence of the non-hydrolyzable ATP analog AMP-PCP to account for the effects of Ca$^{2+}$ and ATP. At low Ca$^{2+}$, there is no change in the FRET efficiency between SERCA2a and PLN in the presence or absence of AMP-PCP at increasing concentrations of CDN1163 vs the DMSO vehicle (Fig. 4B). At high Ca$^{2+}$ conditions, the addition of CDN1163 does not influence the FRET efficiency between SERCA2a and PLN in the presence or absence of AMP-PCP vs. the DMSO vehicle (Fig. 4C). A poor fit of the data to a sigmoid curve, i.e., R$^2$ values <0.18 when fitted to a non-linear concentration-response regression fit, corroborates the inability of CDN1163 to compete with PLN.

Our primary aim was to assess the concentration-response competition between CD1163 and PLN for binding to SERCA2a, and our analysis did not show a sigmoid relationship between CD1163 concentrations and the FRET signal between SERCA2a and PLN. Nonetheless, there may be small, yet significant differences in the SERCA2a-PLN FRET signal at different concentrations of CD1163. To test this, we performed a
two-way ANOVA to establish changes in the FRET signal in response to CDN1163, and
the potential effects of Ca\(^{2+}\) or ATP on the competition between CDN1163 and PLN
compared to the DMSO vehicle. The statistical analysis showed that the FRET
efficiency between SERCA2a and PLN does not significantly change in the presence of
CDN1163 at low (Fig. 5A) or high Ca\(^{2+}\) (Fig. 5B) at all concentrations of the compound,
including those near the half activation (i.e., 2.5 µM, Fig. 5A) and saturating (i.e., 10 and
50 µM, Fig. 5A). Addition of AMP-PCP does not significantly change the SERCA2a–
PLN FRET at functionally relevant concentrations of the compound. These findings
reveal that CDN1163 does not activate SERCA2a by displacing PLN from the regulatory
SERCA2a–PLN complex.

**Effects of ATP on the structural dynamics of SERCA2a**

ATP’s traditional role has been confined as a source of energy to power the Ca\(^{2+}\)-
transporting activity of SERCA, but it has also been suggested that the nucleotide-
binding site plays a role in the so-called ‘distal allostery’ of SERCA.\(^5\) Therefore, we
used TCSPC imaging to investigate whether ATP’s role extends beyond a substrate,
also acting as a modulator of SERCA2a structural dynamics. For these experiments, we
use the non-hydrolysable ATP analog AMP-PCP to eliminate ATP hydrolysis as a
confounding, thus allowing us to isolate the effects of ATP binding on the structural
dynamics of the pump. Since our focus is on the structural shifts that correlate with the
formation of catalytically competent populations, we, therefore, represent the structural
shifts as the percent in the closed population of the headpiece.

We first determined the structural shifts of SERCA in the presence and absence of
AMP-PCP; in addition, we tested different concentrations of thapsigargin (TG), a
selective calcium pump inhibitor,\(^5\) as a control. In the absence of both AMP-PCP and
TG, SERCA2a undergoes a transition toward a higher population of the closed state in
a Ca\(^{2+}\) concentration-dependent manner (Fig. 6A). This Ca\(^{2+}\)-dependent response of
the two-color SERCA2a agrees with previous studies showing that increasing
concentrations of Ca\(^{2+}\) are sufficient to induce a redistribution of structural populations in
the pump.\(^3\) We found that the population of the closed state goes from \(~29.5\%\) at nM
[Ca\(^{2+}\)] concentrations to \(~37.5\%\) at saturating µM of [Ca\(^{2+}\)]. The addition of 0.1 and 1 µM
TG in the absence of AMP-PCP decreases the fraction of the closed population both at
nM and µM Ca\(^{2+}\) concentrations (Fig. 6A); however, this effect is more pronounced at
µM Ca\(^{2+}\) concentrations, where TG significantly decreases the closed population both at
0.1 µM (\(p=0.0001\) ) and 1 µM (\(p=0.0008\) ) of the inhibitor. Yet, TG has no significant effect
on the affinity for Ca\(^{2+}\), e.g., K\(_{Ca}\) values of 1.85 ± 0.19 µM and K\(_{Ca}=1.66 ± 0.34 \) µM in the
absence and presence of 1 µM TG, respectively. The Ca\(^{2+}\)-dependent increase in the
closed population is completely blunted at high (>10 µM) concentrations of TG in the
absence of AMP-PCP (Fig. 6A). This agrees with previous studies have shown that
Ca\(^{2+}\) binding to the pump modulates the structural dynamics of the cytosolic
headpiece.\(^3,\)\(^7,\)\(^31,\)\(^37,\)\(^51,\)\(^53-55\)

SERCA2a also undergoes a structural transition toward a higher population of the
closed state in a Ca\(^{2+}\) concentration-dependent manner upon the addition of AMP-PCP
(Fig. 6B). Addition of AMP-PCP and TG (0.1 and 1 µM) does not decrease the fraction
of the closed population at µM [Ca\(^{2+}\)]. Interestingly, we found that AMP-PCP
substantially increases the fraction of the closed conformation population at nM Ca\(^{2+}\)
concentrations both in the presence and absence of TG (Fig. 6B). The functional
consequences of this effect are illustrated in Fig. 6: In the absence of AMP-PCP, Ca\(^{2+}\)-dependent SERCA2a activation curve reaches a midpoint and a plateau at Ca\(^{2+}\) concentrations of ~2 µM and ~10 µM, respectively. Upon addition of AMP-PCP, the curves reach a midpoint and a plateau at Ca\(^{2+}\) concentrations range of 0.4-0.5 µM and 1-2 µM, respectively. Remarkably, the increase in the fraction of catalytically competent structures predominantly occurs within the physiological Ca\(^{2+}\) concentrations at which SERCA operates (Fig. 6A,B; green shade).

In the presence of AMP-PCP, TG concentrations of 0.1 and 1 µM significantly (p<0.01) decrease the affinity of SERCA2a for Ca\(^{2+}\), showing that AMP-PCP partially reverses the inhibition of Ca\(^{2+}\)-induced structural transitions in the pump. However, the structural transitions induced by AMP-PCP and Ca\(^{2+}\) were completely inhibited at high (>10 µM) concentrations of TG both in the presence and absence of AMP-PCP (Fig. 6A,B). These findings correlate with studies showing that TG inhibits both calcium loading and the formation of catalytically competent SERCA structures. Yet, our findings show more: AMP-PCP is a direct effector of SERCA2a, inducing structural changes in the headpiece of the pump at non-saturating nanomolar Ca\(^{2+}\) concentrations, and partially reversing the inhibitory effects of TG on the population shifts required for activation of SERCA2a. We note that TG fully inhibits SERCA2a’s structural transitions at concentrations higher than 10 µM, but does not shift the fraction of the closed population below 26% (Fig. 6A). We also found that AMP-PCP does not shift the fraction of the closed population above 37% (Fig. 6B). Therefore, these values represent the lower and upper limits between population fractions occupied during Ca\(^{2+}\)- and AMP-PCP-dependent structural transitions of the pump.

**CDN1163 enhances the ATP-mediated modulatory effects to increase the population of catalytically competent SERCA2a structures**

We showed ATP is a modulator of SERCA2a’s structural dynamics, so we tested whether the activator CDN1163 operates through a similar structural mechanism. We first tested whether CDN1163 activates the two-color SERCA2a construct used for the TCSPC imaging experiments. ATPase activity assays using ER microsomes from HEK-293 cells infected with adenovirus showed that the two-color SERCA2a has a V\(_{\text{max}}\) of 0.058 ± 0.001 µmol min\(^{-1}\) mg\(^{-1}\). Incubation of ER microsomes with CDN1163 (5-10 µM) showed that this activator increases the activity of the two-color SERCA2a by 4% relative to the vehicle control. The effect of CDN1163 on the two-color SERCA, while significant (p<0.05), is lower than that observed for the cardiac SR preparation. However, this finding is not unexpected because the ATPase assay is dependent on the protein concentration, and cardiac tissue yields substantially more SERCA2a than the HEK-293 cells. The lower protein yield is consistent with a V\(_{\text{max}}\) of the two-color SERCA2a that is 6-10 times lower than that of the protein isolated from cardiac tissue. Yet, the ATPase activity assays show that the two-color SERCA is catalytically active and that CDN1163 stimulates the activity of this construct.

We next performed TCSPC imaging to show that in the absence of CDN1163 and at low Ca\(^{2+}\) (i.e., [Ca\(^{2+}\)] <0.1 µM), ~28% of the SERCA2a populations are in the closed structure. Increasing Ca\(^{2+}\) concentrations shift the population of the closed structure of SERCA2a to ~37% (Fig. 5). Incubation of SERCA2a with either CDN1163 or AMP-PCP induces a shift in the population of the headpiece toward a closed structure, although the effect is more pronounced in the presence of AMP-PCP (Fig. 7). Interestingly,
incubation of SERCA2a with both CDN1163 and AMP-PCP further shifts the population of the headpiece toward a closed structural state that is comparable to that of SERCA2a alone at saturating Ca²⁺ conditions (Fig. 7). The cooperative effects of CDN1163 and AMP-PCP are more prominent at Ca²⁺ concentrations between 0.1 and 2 µM, although this shift in the structural populations was not observed at [Ca²⁺]>2 µM (Fig. 7). These findings suggest that CDN1163 enhances the Ca²⁺-dependent modulatory effect of ATP to increase the population of catalytically competent SERCA2a structures. We tested this hypothesis by statistical analysis of the changes in the % of the closed headpiece SERCA2a in response to CDN1163, AMP-PCP, and CDN1163/AMP-PCP at twelve Ca²⁺ concentrations.

During cardiac contraction, the intracellular free Ca²⁺ concentration in cardiac cells increases to 1–2.65 µM, allowing interaction between the contractile elements. Relaxation occurs following a decrease in free Ca²⁺ concentrations to 0.1–0.16 µM, causing dissociation of the contractile elements. Therefore, these values represent the upper and lower limits of the range of physiological Ca²⁺ concentrations at which SERCA2a is operational in cardiac cells. The statistical analysis showed that compared to the DMSO vehicle, CDN1163 alone does not have a significant effect on the headpiece population at most Ca²⁺ concentrations tested here (Fig. 8). CDN1163 alone significantly shifts the headpiece populations toward a closed conformation (p<0.05, Fig. 8) at a Ca²⁺ concentration outside the physiological window ([Ca²⁺]=0.001 µM). Compared to the DMSO vehicle, AMP-PCP induces a significant shift in SERCA2a's headpiece toward a closed population at Ca²⁺ concentrations 0.001, 0.01, 0.03, 0.1, 0.56, 1, and 1.8 µM (Fig. 8). The effects of AMP-PCP on SERCA2a agree with previous studies showing that the binding of ATP induces closure of the pump's headpiece. This effect is not significant at a [Ca²⁺]=0.32 µM, which falls within the physiological window of Ca²⁺ concentrations (Fig. 8). AMP-PCP also does not have a significant effect on the headpiece populations at Ca²⁺ concentrations at high Ca²⁺ conditions outside the physiological window (Fig. 8). The most significant effect on the population shift occurs in the presence of both CDN1163 and AMP-PCP at most Ca²⁺ concentrations below 2 µM, e.g., p<0.05 with AMP-PCP vs p<0.01 with AMP-PCP and CDN1163 at a free Ca²⁺ concentration of 1.8 µM (Fig. 8). The treatment of microsomes with both AMP-PCP and CDN1163 does not have a significant effect on the headpiece populations at high (>1.8 µM) Ca²⁺ concentrations that fall outside physiologically relevant Ca²⁺ concentrations (Fig. 8).

Effects of CDN1163 on the affinity of SERCA2a for Ca²⁺ ions

We showed that CDN1163 potentiates the effects of AMP-PCP to produce a population shift required for the activation of the pump. Besides these structural changes, it may be possible that CDN1163 activates SERCA2a by increasing its affinity for Ca²⁺. We tested this mechanism first by analyzing the [Ca²⁺]-dependent ATPase activity curves of SERCA2a at compound concentrations between 0.1–100 µM. We found that at concentrations of CDN1163 equal to or less than 1 µM, the compound does not affect the Ca²⁺ affinity for SERCA, illustrating the fact that concentration-response curves do not shift along the x-axis compared to the control (Fig. 9A). However, at concentrations of CDN1163 equal or higher than 2.5 µM, the concentration-response curves shift to the right along the x-axis.
The ATPase activity curves suggest that while CDN1163 increases the maximal activity of SERCA2a (i.e., turnover rate) (Fig. 9A), it also decreases the apparent SERCA2a’s affinity for Ca$^{2+}$ (Fig. 9B). A limitation of the ATPase activation assays is that the signal depends on hydrolysis of ATP, and therefore, it is impossible to separate the effects of the activator alone or in combination with nucleotide substrate on SERCA2a’s affinity for Ca$^{2+}$. We overcome this limitation by calculating SERCA2a’s affinity for Ca$^{2+}$ directly from the TCSPC imaging curves (Fig. 7). The advantage of this approach is that we eliminate the confounding effects of ATPase hydrolysis. We determined the effect of CDN1163 on SERCA’s K$_{Ca}$ at a concentration of 10 µM because it exerts the maximal stimulatory effect in the ATPase activity assays (Fig. 9A). The calculated K$_{Ca}$ of SERCA2a in the DSMO vehicle and the absence of AMP-PCP is 1.9 ± 0.3 µM, and treatment of the microsomes with CDN1163 significantly increases the K$_{Ca}$ of the pump to a value of 2.7 ± 0.2 µM (p=0.0286, Fig. 9C). In the presence of AMP-PCP, the K$_{Ca}$ of SERCA2a is 1.3 ± 0.2 nM and addition of 10 µM CDN1163 decreases the pump’s affinity for Ca$^{2+}$, with a mean K$_{Ca}$ value 1.5 ± 0.2 nM (Fig. 9D). These findings agree with the ATPase activity assays and indicate that CDN1163 decreases the SERCA2a’s affinity for Ca$^{2+}$. However, SERCA2a activation requires the presence of nucleotide, and the effect of CDN1163 on K$_{Ca}$ is not significant in the presence of AMP-PCP (p=0.8857, Fig. 9D). Collectively, the ATPase assays and TCSPC imaging experiments indicate that CDN1163 does not increase the affinity of SERCA2a for Ca$^{2+}$ ions at functionally relevant conditions of the pump.

**Discussion**

We developed a TCSPC imaging approach to determine the mechanisms for the activation of SERCA2a. This approach, enabled by the recent development of improved fluorescence proteins and hybrid detectors for TCSPC, allows us to detect the individual and combined effects of Ca$^{2+}$, nucleotide substrate, and small-molecule modulators on the structural dynamics of SERCA2a. TCSPC allows the measurement of changes in the fluorescence lifetimes for complex mixtures of fluorescent species. The benefits of this method are a strong statistical background and independence of this method on the concentration of the fluorescence species. The sensitivity of this method is two orders of magnitude more sensitive than methods using concentration-dependent FRET measurements (e.g., acceptor sensitization FRET). Changes in FRET are correlated with changes in the amplitude-weighted average lifetime and together with multiexponential fitting of the whole range of Ca$^{2+}$ concentration enable us to monitor changes in two major fluorescent species. The powerful combination of TCSPC imaging and component analysis yielded structural insights into mechanisms for SERCA2a activation by a small-molecule modulator in unprecedented detail.

We first used ATPase activation assays to screen for SERCA2a effectors that activate SERCA in a concentration-dependent manner. This is important because there are small-molecule probes that exhibit a “bell” or “U-shaped” behavior, which indicates compound-mediated assay interference, and therefore, renders these molecules unsuitable as molecular probes. Among the SERCA effectors reported in the literature, only CDN1163 acts as a direct activator of SERCA2a, increasing its activity sigmoidally, and with an effect on $V_{\text{max}}$ kept at higher concentrations of the compound. These findings agree with a solid-supported membrane biosensing approach showing that CDN1163 enhances SERCA-mediated Ca$^{2+}$ translocation at compound concentrations that are similar to those in this study. An important question we ask is whether the
~12% activation of SERCA2a ATPase activity is functionally significant at the cellular level. We have previously used optical mapping experiments to show that CDN1163 significantly increases Ca\(^{2+}\) dynamics using human iPSC-derived cardiomyocytes.\(^{47}\) These functional effects are comparable to those induced by the adrenergic agonist isoproterenol, which promotes SERCA2a activation via PLN phosphorylation.\(^{14, 60-61}\) These studies validate CDN1163 as a probe to systematically characterize the mechanisms for small-molecule activation of SERCA2a.

PLN inhibits SERCA2a by binding to a large pocket in the transmembrane domain of the pump.\(^{62-67}\) This interaction populates a Ca\(^{2+}\)-ion-free intermediate that serves as a kinetic trap that decreases SERCA’s apparent affinity for Ca\(^{2+}\) and depresses the structural transitions necessary for Ca\(^{2+}\)-dependent activation of the pump.\(^{68-70}\) Therefore, a conventional concept in the field is that dissociation of the SERCA2a–PLN complex is a requirement for SERCA2a reactivation.\(^{48}\) Therefore, we tested whether CDN1163 stimulates the ATPase activity of SERCA2a by displacing PLN from the endogenous SERCA2a–PLN complex. We found CDN1163 does not dissociate the complex even at saturating concentrations of the compound. PLN is thought to interact with Ca\(^{2+}\)-free forms of SERCA and partially dissociate from the pump at high Ca\(^{2+}\) concentrations.\(^{71-72}\) However, CDN1163 has no effect on the FRET signal between SERCA2a and PLN at saturating Ca\(^{2+}\) conditions (i.e., 10 µM). Previous studies have also suggested that PLN and ATP stabilize a functional SERCA–PLN-ATP state that protects the pump against the binding of inhibitors.\(^{73}\) We found that in the presence of the ATP analog AMP-PCP at either low or high Ca\(^{2+}\) conditions, CDN1163 has no effect on the FRET signal between SERCA2a and PLN, showing that CDN1163 does not compete with PLN and has no effect on the PLN/ATP-bound state of the pump. This evidence shows that CDN1163 does not dissociate the SERCA2a–PLN complex, challenging the current paradigm proposing that endogenous PLN needs to be displaced from SERCA to activate the pump.\(^{48-49}\)

We take advantage of the robustness of the TCSPC imaging method, which has over 4 orders of magnitude difference between signal and noise. We complement these experiments with a global analysis of lifetimes for over 200 fluorescence decays and attribute specific lifetimes to structural states of SERCA2a. The combination of TCSPC and global analysis allows us to resolve the structural states of SERCA2a and determine how these states are redistributed in response to ligands. An important advantage of this approach is that we examine the effects of Ca\(^{2+}\), ATP, and small-molecule activators alone or in combination. The use of complementary TCSPC imaging and global analysis approach resolved two primary structural populations of SERCA2a: a catalytically inactive open state, and a closed state that brings together the structural elements required for the formation of a compact headpiece. Indeed, previous studies have shown that the active, disinhibited conformation of SERCA samples is more compact, well-ordered conformations that are catalytically competent.\(^{24, 51, 74}\)

We used this combined approach to first probe the effects of ATP on the dynamics of SERCA2a. We found that in general, the Ca\(^{2+}\)-dependent response resolved by the TCSPC imaging in the presence of AMP-PCP is strikingly similar to that observed in ATPase activity assays, where the midpoint of the curve is observed at Ca\(^{2+}\) concentrations of 0.45–0.5 µM, and the \(V_{\text{max}}\) is usually reached at Ca\(^{2+}\) concentrations of 1–2.5 µM.\(^{29}\) We note that while TCSPC imagining measures structural changes and
ATPase assays measure SERCA2a’s catalytic turnover, the correlation between the two techniques indicates that our approach captures the structural transitions associated with SERCA2a activation. These findings also suggest that ATP primes SERCA2a for activation by increasing the fraction of catalytically competent structures of the pump at physiologically relevant Ca\textsuperscript{2+} concentrations in cardiac cells. ATP and AMP-PCP form virtually identical bound complexes with the pump,\textsuperscript{75} so these findings indicate that ATP is both a substrate and a modulator of SERCA2a dynamics. We then asked whether CDN1163 activates SERCA by a mechanism that is similar to that of ATP. We found that, unlike ATP, CDN1163 alone has no significant effects on the Ca\textsuperscript{2+}-dependent shifts in the structural populations of SERCA2a, and it does not modulate the pump’s affinity for Ca\textsuperscript{2+} ions at functionally relevant conditions. Instead, we found that the combined effect of CDN1163 and AMP-PCP induces a structural shift of the headpiece population that is comparable to that at saturating Ca\textsuperscript{2+} conditions. This population shift occurs only within or below physiological Ca\textsuperscript{2+} concentrations.\textsuperscript{56-58} Based on these findings, we propose that CDN1163 activates SERCA2a by enhancing the ATP-mediated modulatory effects to increase the population of structures that are primed for activation.

Crystallography, spectroscopy, and computational studies have shown that activation and inhibition of SERCA correlate with the shifts between open and closed structural populations of the cytosolic headpiece, the domain of the pump that contains the catalytic elements of the pump.\textsuperscript{32} Specifically, activation of the pump occurs through a population shift toward a closed structure of the headpiece, whereas inhibition by small molecules (e.g., thapsigargin) and endogenous PLN occurs through a shift in the headpiece populations toward an open state.\textsuperscript{32,57,74} While our study correlates well with this mechanism, it shows for the first time how these structural changes occur in response to ligands, nucleotide substrate, and an allosteric modulator quantitatively. This is important because TCSPC experiments at all conditions show that SERCA2a’s headpiece undergoes a maximal ~10% shift in the equilibrium toward the closed state in activating conditions. Relatively small shifts in structural populations are a common theme in the allosteric modulation of sarcomeric proteins. For example, allosteric activation of the smooth muscle myosin induced by phosphorylation of its regulatory light chain occurs with a ~20% shift in the structural populations within its phosphorylation domain.\textsuperscript{76}

In conclusion, in this work we introduce TCSPC imaging and global analysis of fluorescent lifetimes, a powerful approach that makes possible the direct analysis of the structural mechanisms underlying allosteric modulation of proteins. A key feature of this combined approach is the resolution of protein structural states and population shifts in response to ligands, substrates, and small molecules. This technical advantage allowed us to monitor the structural mechanisms for activation of the cardiac calcium pump SERCA2a by CDN1163, a validated small-molecule allosteric modulator. The significance of this space-time resolution is threefold. First, the experiments directly showed that CDN1163 does not compete for binding with SERCA2a’s endogenous regulator PLN and that this effect is independent of Ca\textsuperscript{2+} and ATP. Second, the method allowed us to directly measure the response of SERCA2a to ligands and show that CDN1163 does not activate SERCA2a by modulating the pump’s affinity for Ca\textsuperscript{2+}. Finally, we showed that CDN1163 and ATP act synergistically to populate SERCA2a structures that are primed for ATP utilization and SERCA2a’s phosphorylation. In summary, this study provides novel insights into the synergy between SERCA2a’s
substrate and a synthetic allosteric modulator to activate a clinically important target in
the heart.

Acknowledgments
We thank Gail Rising, Coordinator of the Animal Surgery Operating Rooms at the
University of Michigan Unit Laboratory Animal Medicine, for the donation of pig hearts.
We thank Sean Cleary for the optimization of Ca\(^{2+}\)-containing buffers, and Ellen Cho for
technical assistance. J.S. was supported by an American Heart Association Postdoctoral Fellowship 830562. This work was supported by grants from the National Institutes of Health R01GM120142 and R01HL148068 (to L.M.E.-F.), and R01HL092321 and R01HL143816 (to S.L.R.). This research was supported in part through computational resources and services provided by Advanced Research Computing, a division of Information and Technology Services at the University of Michigan, Ann Arbor. this manuscript was posted on a preprint: https://doi.org/10.1101/2023.09.07.556734.

Declaration of Interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability
All data are available in the main text.

Author Contributions
Jaroslava Seflova: Designed research; Performed research; Analyzed data.
Carlos Cruz-Cortes: Performed research; Analyzed data.
Guadalupe Guerrero-Serna: Performed research; Analyzed data.
Seth Robia: Analyzed data.
L. Michel Espinoza-Fonseca: Designed research; Analyzed data; Wrote the paper.

References


71. Asahi, M.; McKenna, E.; Kurzydlowski, K.; Tada, M.; MacLennan, D. H., Physical interactions between phospholamban and sarcoplasmic reticulum Ca2+-ATPases are dissociated by elevated Ca2+, but not by phospholamban phosphorylation, vanadate, or thapsigargin, and are enhanced by ATP. The Journal of biological chemistry 2000, 275 (20), 15034-8.

72. Chen, Z.; Akin, B. L.; Jones, L. R., Ca2+ binding to site I of the cardiac Ca2+ pump is sufficient to dissociate phospholamban. J Biol Chem 2010, 285 (5), 3253-60.


**Fig. 1.** Overview of the TCSPC imaging approach to probe the mechanisms for SERCA2a activation. (A) Snapshot of the modeled 3D structure of the two-color cardiac SERCA2a construct labeled with a maroon fluorescent protein (mMaroon) at position Met1 and with an orange fluorescent protein (OFP) at position Gly509. The approximate location of the lipid bilayer is represented by the shaded gray box. We note that this structure is shown for representation only. (B) Fluorescence decay measured by TCSPC for two-color SERCA2a in the DMSO vehicle. We found that the TCSPC imaging approach has over 4 orders of magnitude difference between signal (intensity at ~10^6 AU) and noise (intensity at ~10^-4 AU).

**Fig. 2.** Effects of small-molecule effectors on SERCA2a ATPase activity. (A) Structures of chemically diverse small-molecule effectors reported in the literature as SERCA activators. 10-point concentration-response curves were obtained for (B) CDN1163, (C) istaroxime, (D) CP-154526, and (E) Ro 41-0960 on the ATPase activity of the cardiac SERCA2a. In all cases, the activity of the compounds at each concentration was obtained from an 8-point free Ca^2+ concentration-dependent SERCA2a activity assay and normalized relative to the untreated control, as described in the experimental procedures. Data are reported as average ± SEM of three biological replicates (N=3).

**Fig. 3.** PLN removal does not affect the catalytic turnover of SERCA2a. (a) Western blot analysis was used to detect the presence of PLN along the purification steps of SERCA2a, starting from pig cardiac SR microsomes. The Western blot analysis showed that purification of SERCA2a removes PLN initially present in the pig cardiac SR microsomes. (b) Ca^2+ concentration-dependent ATPase activity plot of purified SERCA2a. The Vmax calculated from the fitted data is shown inside the plot. Data are reported as the average ± SEM of four biological replicates (N=4).

**Fig. 4.** Competitive FRET assays to investigate the effects of CDN1163 on the dissociation of the endogenous SERCA2a–PLN complex. (A) Structural representation of the dissociation and subunit models for activation of SERCA2a by CDN1163 tested in this study. Concentration-response curves at increasing CDN1163 concentrations under (B) low free Ca^2+ and (C) high free Ca^2+ conditions. For comparison, we tested the effects of AMP-PCP alone or in the presence of CDN1163. Data are reported as the average ± SEM of four biological replicates (N=4).

**Fig. 5.** Effects of CDN1163 on the interaction between SERCA2a and PLN. FRET assays were performed at (A) low Ca^2+ conditions (0.01 µM) and (B) high Ca^2+ conditions (10 µM). In all cases, we found that compared to the DMSO vehicle, increasing concentrations of CDN1163 (red numbers) do not significantly change the FRET efficiency between fluorescently labeled SERCA2a and PLN. The addition of the ATP analog AMP-PCP to the SERCA2a- and PLN-containing microsomes does not induce significant changes in the FRET signal vs the control at both low and high Ca^2+ conditions. Treatment of the microsomes with a fixed concentration of AMP-PCP and increasing concentrations of CDN1163 does not significantly change the FRET efficiency compared to the vehicle, CDN1163, and AMP-PCP treatment groups. Data is shown as mean ± SEM of four biological replicates (N=4). We used a two-way ANOVA followed by the Dunnett’s post-hoc test to compare treatments (CDN1163, AMP-PCP, and CDN1163+AMP-PCP) against the DMSO vehicle; ns=not significant.
Fig. 6. Structural changes of SERCA2a in response to the ATP analog AMP-PCP and the inhibitor thapsigargin. 12-point Ca\(^{2+}\) concentration-response curves were obtained in the (A) absence and (B) presence of AMP-PCP at four concentrations of thapsigargin. In both cases, we include a DMSO vehicle as a control. The structural change in response to ligands is shown as the % of the closed population of the headpiece. The dashed lines show the lower, midpoint, and upper boundaries of the concentration-response curve of SERCA2a in the absence of AMP-PCP and TG. The green shaded area highlights the range of Ca\(^{2+}\) concentrations at which SERCA2a is operational in cardiac cells. Data are reported as average ± SEM of four biological replicates (N=4).

Fig. 7. Structural changes of SERCA2a in response to CDN1163. 12-point Ca\(^{2+}\) concentration-response curves were obtained in the presence of DMSO vehicle (solid black trace), CDN1163 (dashed black trace), AMP-PCP (solid red trace), and CDN1163+AMP-PCP (dashed red trace). The structural change in response to ligands is shown as the % of the closed population of the headpiece. Data are reported as average ± SEM of four biological replicates (N=4).

Fig. 8. Effects of CDN1163, AMP-PCP, and CDN1163/AMP-PCP on the structural populations of SERCA2a. TCSPC assays were performed at increasing Ca\(^{2+}\) conditions from 0.001 µM to 100 µM. Intracellular free Ca\(^{2+}\) concentration in cardiac cells fluctuates in the range of 1–2.65 µM and 0.1–0.16 µM during contraction and relaxation of the heart. Therefore, these values represent the upper and lower limits of the range of physiological Ca\(^{2+}\) concentrations at which SERCA2a operates. The full range of Ca\(^{2+}\) concentrations used in this study is shown, where the physiological window is shown in green, and low and high Ca\(^{2+}\) concentrations falling outside this window are shown in blue and red, respectively. In all cases, we used AMP-PCP and CDN1163 concentrations of 0.5 and 10 µM, respectively. Data is shown as mean ± SEM of four biological replicates (N=4). We used a two-way ANOVA followed by the Dunnett's post-hoc test to compare treatments (CDN1163, AMP-PCP, and CDN1163/AMP-PCP) against the DMSO vehicle. *p<0.05; **p<0.01; ***p<0.001; **** p<0.0001.

Fig. 9. Effects of CDN1163 on SERCA2a’s affinity for Ca\(^{2+}\) ions. (A) 8-point Ca\(^{2+}\) concentration-response of SERCA2a ATPase activity curves obtained at ten concentrations of CDN1163 (0.1 to 100 µM). The activity at each concentration of CDN1163 was normalized relative to the control at [Ca\(^{2+}\)]\(_{0}\) =1 µM because this is the point representing the maximal activity of the pump in our assays. (B) Changes in apparent Ca\(^{2+}\) affinity (K\(_{Ca}\)) induced by increasing concentrations of CDN1163; the apparent affinity is expressed as the %change relative to the vehicle control. Data are reported as average ± SEM of three biological replicates (N=3). To eliminate the confounding effects of ATPase hydrolysis, we used TCSPC imaging to measure the effects of 10 µM CDN1163 on SERCA2a’s K\(_{Ca}\) in the (C) absence and (D) presence of the ATP analog AMP-PCP. Data is shown as mean ± SEM of four biological replicates (N=4). Significance compared to the DMSO vehicle was determined using a two-tailed Mann–Whitney U test; *p<0.05.
Figure 1
270x136 mm (DPI)

Figure 2
344x188 mm (DPI)
Figure 3
408x147 mm (DPI)
Figure 4
213x199 mm (DPI)
Figure 5
333x230 mm (DPI)

Figure 6
341x152 mm (DPI)
Figure 7
201x140 mm (DPI)

Free Ca\(^{2+}\) concentration (μM)

- DMSO
- CDN1163
- AMP-PCP
- AMP-PCP+ CDN1163

Figure 8
449x245 mm (DPI)
Figure 9
450x112 mm (DPI)