Solid-state NMR and Functional Measurements Indicate That the Conserved Tyrosine Residues of Sarcolipin Are Involved Directly in the Inhibition of SERCA1*

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The transmembrane protein sarcolipin regulates calcium storage in the sarcoplasmic reticulum of skeletal and cardiac muscle cells by modulating the activity of sarco(endo)plasmic reticulum Ca2+-ATPases (SERCAs). The highly conserved C-terminal region (2RSYQY-COOH) of sarcolipin helps to target the protein to the sarcoplasmic reticulum membrane and may also participate in the regulatory interaction between sarcolipin and SERCA. Here we used solid-state NMR measurements of local protein dynamics to illuminate the direct interaction between the Tyr29 and Tyr31 side groups of sarcolipin and skeletal muscle Ca2+-ATPase (SERCA1a) embedded in dioleoylphosphatidylcholine bilayers. Further solid-state NMR experiments together with functional measurements on SERCA1a in the presence of NAc-RSYQY, a peptide representing the conserved region of sarcolipin, suggest that the peptide binds to the same site as the parent protein at the luminal face of SERCA1a, where it reduces Vmax for calcium transport and inhibits ATP hydrolysis with an IC50 of ~200 μM. The inhibitory effect of NAC-RSYQY is remarkably sequence-specific, with the native aromatic residues being essential for optimal inhibitory activity. This combination of physical and functional measurements highlights the importance of aromatic and polar residues in the C-terminal region of sarcolipin for regulating calcium cycling and muscle contractility.

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The abbreviations used are: SR, sarcoplasmic reticulum; CP-MAS, cross-polarization magic-angle spinning; SLN, sarcolipin; SERCA1a, the fast-twitch skeletal muscle isoform of the sarco(endo)plasmic reticulum Ca2+-ATPase; PLB, phospholamban; DOPC, dioleoylphosphatidylcholine; BAPTA, 1,2-bis(2-aminoxyethoxy)ethane-N,N,N',N'-tetraacetic acid (tetrasodium salt); PIPES, 1,4-piperazinediethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DIPSHIFT, dipolar-chemical shift correlation; l/p, lipid to protein.

Normal physiological control of muscle contractility depends on the strict regulation of intracellular calcium cycling (1). Muscle relaxation is coupled to the reuptake of Ca2+ by the sarcoplasmic reticulum (SR),2 driven by the sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA), a 110-kDa membrane-embedded ion pump that maintains a 10,000-fold Ca2+ concentration gradient across the SR membrane (2). The cardiac isoform of SERCA (SERCA2a) associates reversibly with the pentameric transmembrane protein phospholamban (PLB) in the SR membrane resulting in a transitory inhibition of calcium transport into the SR, which is essential for efficient muscle contraction. Inhibition of SERCA2a is relieved during muscle relaxation as a result of phosphorylation of PLB by cAMP-dependent protein kinases in response to β-adrenergic stimulation.

Sarcolipin (SLN) is a 31-amino acid PLB homologue that co-expresses and co-localizes with the fast twitch skeletal muscle (SERCA1a) isoform of Ca2+-ATPase and, to a lesser extent, with SERCA2a in cardiac cells (3–5). SLN causes a decrease in the apparent affinity of SERCA enzymes for calcium, but its effect on Vmax is less clear, with some studies noting an inhibitory effect and others showing a stimulatory response (6, 7). Expression of SLN in cardiac tissue is low in humans and predominates in the atria, whereas PLB levels are higher and localized in the ventricles, but there is mounting evidence that SLN plays a role in regulating calcium cycling in healthy and compromised heart muscle (10–13). Co-expression of SERCA2a with SLN and PLB enhances the inhibition of calcium transport by SERCA2a, resulting either from the formation of a superinhibitory binary complex or from an SLN-induced shift in the monomer-pentamer equilibrium of PLB (8, 9).

SLN has a compact α-helical transmembrane domain, extending from Arg6 through Arg27, which is oriented approximately perpendicular to the plane of the membrane (14) with the C terminus facing toward the SR lumen. The C-terminal region contains five perfectly conserved C-terminal amino acids, 2RSYQY, which lie outside the lipid bilayer and appear to act as a signal sequence that targets SLN to the SR membrane (15). Recent NMR studies of SLN in detergent micelles have indicated that the polypeptide backbone around the dynamic C terminus becomes more constrained in the presence of SERCA, which implies that this region of the SLN backbone may be stabilized through interactions with the enzyme (14, 16). Co-expression of null-tyrosine mutants of FLAG-tagged SLN with SERCA in HEK-293 cells resulted in alterations in Vmax and KCa for calcium uptake by SR microsomes relative to preparations...
with wild-type SLN, suggesting that Tyr29 and Tyr31 of SLN may play some role, either direct or indirect, in modulating the function of SERCA (6). Mutations of the tyrosine residues could, however, influence various features of SLN within the cell, such as expression levels and membrane targeting (15) or oligomeric status, overall topology, and structure, any of which could influence indirectly the effect that SLN has upon SERCA function. Hence it is unclear whether Tyr29, Tyr31, and other residues in the conserved C-terminal region of SLN regulate SERCA as a result of direct contact with the enzyme.

This work has applied solid-state nuclear magnetic resonance (SSNMR) in alliance with conventional biochemical methods to investigate whether the conserved tyrosine residues of SLN participate in a regulatory interaction with SERCA1a within phospholipid membranes. From a combination of structural, dynamic, and functional measurements of SLN and SERCA1a, it is shown that Tyr29 and Tyr31 of SLN make contact with the luminal face of the enzyme and that all five of the residues RSYQY contribute toward the inhibition of ATP hydrolysis and calcium transport. The work also provides some evidence that the luminal region of SLN together with the cytoplasmic domain of PLB inhibits SERCA1a more effectively than either region alone, consistent with the superinhibitory effect observed when SLN and PLB are co-expressed with the enzyme (9). Our findings provide new insights into the regulation of skeletal muscle contractility by SLN and, in view of the close similarities of SERCA1a and SERCA2a, are also likely to be relevant to the regulation of cardiac calcium cycling.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic SLN (>95% pure) was purchased from Activotec SPP Ltd., University of Southampton. The protein was uniformly 13C- and 15N-labeled at Tyr29 and uniformly 13C-labeled at Tyr31. A second SLN sample (>95% pure) was labeled with 2H at the α-position of Val20, [α-13C-Tyr]NAc-RSYQY and unlabeled derivatives were purchased from Peptide Protein Research Ltd., Fareham, UK.

The detergent n-octyl β-d-glucopyranoside (OG) was purchased from Melford Laboratories, and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (tetrosodium salt) (BAPTA) was purchased from Molecular Probes. All other chemicals were purchased from Sigma.

**Preparation of SR Microsomes and Further Purification of SERCA1a**—SERCA1a was purified from fast-twitch rabbit skeletal muscle according to a method adapted from East and Lee (17). First, SR microsomes were prepared from 100 g of muscle tissue by differential centrifugation. A fraction of the intact SR vesicles was retained for calcium uptake measurements, and the remainder was treated with sodium cholate and subjected to density gradient centrifugation to yield membranes containing ~100 mg of ~90% pure SERCA1a as characterized by SDS-PAGE on a 10% resolving gel. Protein concentrations were calculated using an adaptation of the Lowry method (18).

**Reconstitution of SERCA1a and SLN**—Purified SERCA1a was reconstituted into dioleoylphosphatidylcholine (DOPC) membranes at a lipid to protein (l/p) molar ratio of 160:1, using an adaptation of methods described previously (19–21). SLN was co-reconstituted with SERCA1a into DOPC membranes at a lipid/SLN/SERCA1a molar ratio of 160:10:1 or 160:3:1 (21). For the reconstitution of SLN alone, DOPC and SLN were dissolved in 50:50 chloroform/methanol at an l/p molar ratio of 53:1 and dried to a thin film as described above. Samples were rehydrated in 10 mM phosphate, pH 7, and centrifuged at 14,000 rpm in a bench top microcentrifuge. SLN was visualized on Coomassie Blue-stained 15% Tris-Tricine gels according to the method of Schagger and von Jagow (22). Apparent molecular weights were determined using ultra low molecular weight markers (1,06, 3,5, 6,5, 14,2, 17, and 26.6 kDa).

**Activity Measurements**—Specific Ca2+-ATPase activity was quantified as the amount of inorganic phosphate (Pi) liberated upon ATP hydrolysis at 37 °C, as described previously (21). Ca2+ uptake by SR microsomes was measured on a Carey Eclipse fluorimeter by monitoring the change in fluorescence of the calcium indicator BAPTA in a method adapted from Refs. 20, 23. SR microsomes were added to Ca2+ uptake buffer (10 mM PIPES, 5 mM MgSO4, 100 mM K2HPO4, pH 7.1) containing 50 μM BAPTA and 0.25 μM carbonylcyanide p-trifluoromethoxyphenylhydrazone and CaCl2 to the desired final calcium concentration in an assay volume of 2 ml. The final protein concentration was 20 μg/ml. The reaction was initiated with ATP to a final concentration of 4 mM, and the fluorescence was monitored at an emission wavelength of 370 nm after excitation at 299 nm, over a time course of 5–10 min. SR microsomes were opened and reformed in the presence of NAc-RSYQY according to the following procedure. A 0.5-ml volume of SR (2 mg/ml protein) in Ca2+ uptake buffer was incubated on a shaker at room temperature overnight in the presence of 1 mg/ml C12E8 (control) or 1 mg/ml C12E8 plus 200 μM NAc-RSYQY. 200 mg of washed and equilibrated XAD2 resin was then added to remove the detergent. Samples were returned to the shaker for a further 4 h before carrying out Ca2+ uptake measurements. (C12E8 was omitted from samples used for measuring the effect of NAc-RSYQY on the cytoplasmic domain of Ca2+ ATPase.)

Free calcium was calculated from a method adapted from Tatulian et al. (24), using the calculation [Ca2+]i = Kd × (Fi − F)/Fi − F, where F is the BAPTA fluorescence measured and substracts f, i, and s represent the fluorescence for free calcium, the ith addition of calcium, and the saturating level of calcium, respectively. A Kd value of 203 nm was assumed for the calculations. Fluorescence values were taken during the uptake assay using measurements after the addition of known amounts of calcium and before addition of ATP to initialize the reaction. The fluorescence values obtained were substituted into the above equation, and the linear part of the resulting graph was used to convert added calcium to free calcium. Membranous Na+/K+-ATPase was purified from pig kidney microsomal membranes, and enzymatic activities and protein concentrations were determined as described previously (25, 26).

**Electron Microscopy**—SR samples (50 μl) at a protein concentration 100 μg/ml in PIPES buffer, before and after treatment with C12E8 and/or Bio-Beads, were applied to glow-discharged 400 mesh carbon-coated copper grids. A negative stain (2% uranyl acetate) was applied prior to visualizing by electron microscopy using a Tecnai 10 microscope.

**Solid-state NMR Measurements**—NMR experiments were performed using a Bruker Avance 400 spectrometer operating
at a magnetic field of 9.3 tesla. The sample temperature was 4 or 30 °C. The diameter of the NMR sample rotor was 4 mm, and the spinning rate ranged from 4 to 8 kHz, maintained automatically to within ± 1 Hz. Typical conditions for CP-MAS experiments were a 1H 90° excitation pulse length of 4.0 μs, Hartmann-Hahn cross-polarization from 1H to 13C at a contact time of 1–5 ms using a matched 1H field of 65 kHz, continuous wave proton decoupling at a field of 85 kHz during signal acquisition, and a 2-s recycle delay. Two-dimensional dipolar assisted rotational resonance spectra (27) were recorded with 128 hypercomplex points in the indirect dimension with a mixing time of 50 ms during which the proton field was adjusted to the spinning frequency of 8 kHz. Two-dimensional C-H DIPSHIFT spectra were recorded using the constant time experiment described elsewhere (14), at a spinning rate of 7822 kHz. The indirect dimension of the two-dimensional spectra was constructed as follows. A series of n = 8 free induction decays was recorded, each after allowing the 13C magnetization to evolve under the influence of C-H dipolar interactions for up to 1 cycle of sample rotation (t c) at eight regular intervals t incremented by 18.6 μs, where 0 ⩽ t ⩽ t c. In practice this was achieved using frequency-switched Lee-Goldberg homonuclear decoupling during the evolution interval. The free induction decays were Fourier-transformed into 1024 points, and the intensity values at each equivalent point in the eight spectra were normalized to the value at t = 0 and corrected for T 2 relaxation by multiplication of each point by a factor exp(t/T 2), substituting a value of T 2 such that the intensity values at t = 0 and t = 8 were equal to 1. The eight spectra (S n) were then replicated and concatenated (S 1, S 2, ... S 8, S 9, S 10, ... S 16, etc) to give a 1024 × 128 matrix, which was Fourier-transformed in the indirect dimension to produce the pseudo two-dimensional spectra, with the 13C chemical shift information in the direct dimension and C-H dipolar coupling information in the indirect dimension. C-H dipolar spectra corresponding to different sites in SLN were obtained as vertical slices through the two-dimensional spectra at specific chemical shifts. Simulations of the C-H spectra in terms of the rate of rotational diffusion of SLN or the aromatic ring dynamics of Tyr29 and Tyr31 were carried out as described previously (28). The 2H NMR spectrum of [α-2H-V20]SLN in DOPC bilayers was obtained with MAS at 12.5 kHz using the quadrupole echo experiment (29) with the two delays τ 1 and τ 2 set to one full period of sample rotation.

RESULTS

Preliminary Characterization of SLN—In previous work it was shown that SLN is capable of forming tetramers, pentamers, and possibly higher aggregates in intact liposomes (30). Here, the oligomeric state of SLN in DOPC membranes at an l/p molar ratio of 53:1 was explored using NMR measurements of molecular dynamics. The rate of rotational diffusion of SLN within the lipid bilayer is inversely proportional to the cube of the transmembrane diameter of the rotating species. Synthetic SLN, with the α-position of residues Tyr29 and Tyr31 labelled with 13C and the α-position of Val19 labelled with 2H, was incorporated into DOPC membranes, and the overall rotational mobility of SLN was probed using 13C and 2H solid-state magic-angle spinning (MAS) NMR experiments. The spectra are sensitive to motional averaging of the 2H quadrupolar anisotropy or the 1H-13C dipolar anisotropy as a result of rotational diffusion of SLN with a rotational correlation time τ 2SLN in the range 10−8–10−6 s. The spectra indicated that neither the dipolar and quadrupolar anisotropies were scaled markedly by motional averaging (Fig. 1, a and b), suggesting either that τ 2SLN was slower than 10−4 s or that the C-2H and C-H bonds were oriented so as to be insensitive to rapid rotational diffusion (as demonstrated by the simulated line shapes in Fig. 1, a and b). It is calculated that the transmembrane diameter of a species with τ 2SLN > 10−4 s is on the order of 50 Å or more (31). By comparison, PLB pentamers have a transmembrane diameter of ∼30 Å (32), so it follows that here the NMR data are consistent with SLN aggregates with an oligomeric number of much greater than 5. Detergent-solubilized SLN ran as a monomer on SDS-polyacrylamide gels, however, and bands corresponding to larger species were not observed (Fig. 1c) suggesting that any oligomers present were not stable in detergent. Moreover, when a sample of SLN in DOPC membranes (at an l/p molar ratio of 53:1) was treated with the cross-linking agent p-azidophenylglyoxal monohydrate as described previously (30), Tris-Tricine gels again showed no evidence of cross-linked oligomers (data not presented). Given that the presence of oligomers could not be confirmed by biochemical methods, an alternative interpretation of the NMR data is that SLN remains monomeric but tilted in the membrane with the transmembrane helix rotated to allow the C-2H and 13C-H bonds to make angles of close to 0° with respect to the axis of molecular rotation (usually parallel with the bilayer normal). Rapid rotation of the SLN monomer about this axis would not then average the dipolar and quadrupolar anisotropies. Such a model supports recent experiments that showed SLN to be tilted by ∼23° in the membrane (33).

Interactions between SLN and SERCA1a—NMR and functional experiments were next carried out to establish whether SLN and SERCA1a interact with each other in the DOPC membranes. ATP hydrolysis measurements indicated that reconstituted SERCA1a retained calcium-dependent activity, but in the presence of a 3-fold excess of SLN there was a slight reduction in V max. When in a 10-fold excess over SERCA1a, SLN shifted the activity curve to the right and depressed V max by about 25%, indicating that high concentrations of SLN lowered both the calcium affinity and maximal rate of transport of SERCA1a (Fig. 2a). The functional information therefore implies that a physical interaction takes place between the two proteins in membranes suitable for NMR analysis.

The interaction between SLN and SERCA1a was probed from the perspective of the conserved tyrosine residues Tyr29 and Tyr31 of SLN within DOPC membranes (at an l/p ratio of 53:1) using 13C SSNMR methods to observe the physical and dynamic properties of the tyrosine rings in the presence and absence of SERCA1a. Peaks in the 13C spectrum of DOPC membranes containing [U-13C-Tyr29/Tyr31]SLN were assigned to the two tyrosine aromatic spin systems by inspection of two-dimensional 13C-13C dipolar correlation spectra (Fig. 2h, black spectrum). The peaks for the e-carbon site for Tyr29 and Tyr31, at ∼120 and ∼115 ppm, respectively, were almost fully resolved in the one-dimensional spectrum, as were the peaks for the
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![Image](https://via.placeholder.com/150)

**FIGURE 1.** Experiments to probe the dynamics and oligomeric state of SLN in SDS micelles and in DOPC membranes at 30 °C. The dynamics of SLN in DOPC membranes at an l/p molar ratio of 53:1 were examined by measuring the rate of SLN rotational diffusion at 30 °C using magic-angle spinning NMR methods to observe the 1H-13C dipolar anisotropy for the α-positions of [U-13C-Tyr29/Tyr31]SLN or the 2H quadrupolar line shape for [α-2H-Va1]-SLN (a). The experimental 1H-13C dipolar spectrum was obtained from the projection of the indirect dimension of a 1H-13C two-dimensional DIPSHIFT spectrum in the Cα region (b). Experimental spectra are compared with simulations for different protein rotational correlation times (τR) and C-H and C-D bond orientations (c). The experimental spectra agree well with simulated spectra for τR of >10−4 s if the C-H and C-D bonds are oriented at an angle of 60° relative to the axis of protein rotational diffusion (b, bottom), which occurs when the protein is aligned parallel to the bilayer normal. The experimental spectra also agree with simulated spectra for τR of <10−5 s if this is zero (b, top), which can occur if the protein is tilted in the membrane. SDS-polyacrylamide gels of SLN (25 μg/μL of sample buffer) show a single band consistent with the size of monomeric protein (d). Single bands consistent with monomers are also seen on gels of SLN reconstituted into DOPC membranes at l/p molar ratios of 10:1, 20:1 and 53:1 and treated with the cross-linking agent azidophenylglyoxal monohydrate (e).

ζ-carbon sites of the two tyrosines. Small changes in the tyrosine side group chemical shifts and a broadening of the peaks were observed when SLN was reconstituted in a 3-fold excess over SERCA1a (Fig. 2b, blue spectrum), suggesting that Tyr29 and Tyr31 undergo conformational changes or experience a change in the microenvironment when the enzyme is present in the membrane. In the presence of SERCA1a several of the peaks for the aromatic residues (including the Tyr31ε cross-peak) appear to be split, consistent with SLN existing in multiple environments or physical states. One explanation for the peak splitting is that the spectrum represents free and SERCA-bound species of SLN in slow exchange. Alternatively, peak splitting could occur if SLN undergoes a slow transformation between different conformations induced by the presence of SERCA1a. NMR experiments on SLN and PLB in oriented lipid bilayers and detergent micelles by Veglia and co-workers (33, 34) have identified multiple conformations or topologies of both proteins. The authors postulated the presence of a pre-existing equilibrium between so-called R and the T conformational states apparent in the cytoplasmic domain of PLB prior to SERCA binding (35), and suggested that the dynamics of the SLN transmembrane domain may facilitate a similar topological interconversion of SLN that could be important for recognition by SERCA. The spectra obtained here support the existence of two or more conformations of SLN in unoriented lipid bilayers that are reflected partially in the C-terminal region of the protein.

Specific associations of the tyrosine side groups with residues within SERCA1a might impaire the mobility of the aromatic rings. A two-dimensional C-H DIPSHIFT experiment was therefore used to measure the 1H-13C dipolar anisotropy for the aromatic carbons of Tyr29 and Tyr31 to specifically monitor the dynamics of the SLN tyrosine rings in the absence and presence of SERCA1a. The dipolar anisotropy at the ortho- and meta-positions of the aromatic rings is sensitive to the rate of ring reorientation resulting from rotation about the Cβ-Cγ bond. Experimental spectra for [U-13C-Tyr29/Tyr31]SLN in the absence and presence of SERCA1a were compared with simulated spectra calculated for different rates of ring reorientation. The entire aromatic range of the spectrum was simulated, but in principle the 1H-13C anisotropy measured only for the ortho-ring positions (i.e. the ε-positions of Tyr29 and Tyr31) would be sufficient to estimate the rates of rotation of the two rings. Spectra of SERCA1a reconstituted alone in DOPC membranes exhibited negligible signal intensity in the aromatic region. The results indicated that, in the absence of SERCA1a, the rings of both Tyr29 and Tyr31 undergo rapid rotational motion with a local correlation time (τK) of shorter than 10−5 s (Fig. 3, top). In the presence of the enzyme, however, the mobility of both the Tyr29 and Tyr31 rings was impaired significantly, lengthening τK to greater than 10−5 s (Fig. 3, bottom). This observation, taken together with the changes in the aromatic 13C chemical shifts observed in the presence SERCA1a, suggests that Tyr29 and Tyr31 of SLN make contact with SERCA1a. These results prompted further experiments to confirm that an interaction occurs between the C-terminal residues of SLN and the enzyme.

**Regulation of SERCA1a by SLN Peptide Fragments**—The interaction between Tyr29 and Tyr31 of SLN and SERCA1a, when considered alongside the high degree of conservation
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**FIGURE 2. Detection of interactions between SERCA1a and SLN in DOPC bilayers from functional and physical measurements.** Calcium-dependent ATPase activity of SERCA1a was determined in the absence of SLN (filled squares) and at a SLN/SERCA1a molar ratio of 3:1 (open circles) and 10:1 (open triangles). Each measurement used 10 μg of SERCA1a, and the DOPC/SERCA1a molar ratio was 160:1. The maximal activity of SERCA1a in the absence of SLN was 1.5 mmol/mg per min. The role of the tyrosine residues of SLN (Tyr29 and Tyr31) in the protein-protein interaction was assessed from the aromatic region of a two-dimensional 13C dipolar assisted rotational resonance spectrum of [U-13C-Tyr29/Tyr31]SLN alone in DOPC membranes at a l/p molar ratio of 53:1 (black) or in the presence of SERCA1a at a DOPC/SLN/SERCA1a molar ratio of 160:3:1 (blue) at 30 °C (b). The spectra exhibit distinct peaks for the e and γ carbons of Tyr29 and Tyr31, and generally show changes in the aromatic 13C chemical shifts in the presence of SERCA1a, consistent with alterations in the SLN tyrosine ring orientations and/or microenvironment in the presence of enzyme.

**FIGURE 3. NMR experiments to detect the interaction between the Tyr29 and Tyr31 side groups of [U-13C-Tyr29/Tyr31]SLN with SERCA1a by measuring the rotational dynamics of the tyrosine aromatic rings.** The correlation times for ring rotation (τ_e and τ_γ) will lengthen if the side groups are restrained by residues within SERCA1a, as illustrated by the model of the SLN-SERCA1a complex (viewed from the luminal face) shown on the left. [U-13C-Tyr29/Tyr31]SLN was incorporated into DOPC membranes alone at an l/p molar ratio of 53:1 (top panels) or in the presence of SERCA1a at an SLN/SERCA1a molar ratio of 3:1 (bottom panels) at 30 °C. Correlation times were estimated by comparing the aromatic region of experimental 1H-13C two-dimensional DIPSHIFT spectra for [U-13C-Tyr29/Tyr31]SLN (left panels) with simulated two-dimensional DIPSHIFT spectra calculated for different values of τ_e and τ_γ. Shown at the right of each spectrum are cross-sectional slices through the DIPSHIFT spectrum at the positions shown, representing dipolar spectra for the e-C-H groups of Tyr29 (red) and Tyr31 (black). Comparison of the experimental spectra (left) with the closest matching simulations (right) indicate that the experimental spectra are consistent with the two rings undergoing rapid rotational reorientation (on the C-H dipolar time scale) in the absence of SERCA1a (τ_e < 10^−5 s and τ_γ < 10^−5 s) and with the rates of rotation being reduced significantly in the presence of the enzyme (τ_e > 10^−5 s and τ_γ > 10^−5 s).

seen for the C-terminal amino acids, suggests that the aromatic residues and perhaps the entire conserved RSYQY-COOH sequence might play a role in modulating the function of SERCA enzymes. To assess the inhibitory properties of the conserved sequence independently of the transmembrane region of SLN, the water-soluble pentapeptide NAc-RSYQY-COOH (N-terminally acetylated to preserve the native charge of Arg27 in SLN) was screened for inhibition of ATPase activity when added to SERCA1a purified in a native membrane preparation. The peptide abolished ATP hydrolysis with an IC_{50} of around 200 μM (Fig. 4a). The same peptide concentrations had no effect on the SERCA homologue, Na+/K⁺-ATPase, suggesting that NAc-RSYQY could be specific for the calcium pump. A NAc-RSYQY concentration of 100 μM lowered V_{max} for calcium-dependent ATP hydrolysis but had a negligible effect upon calcium affinity for the enzyme (Fig. 4b, circles). Thus, these experiments provided a preliminary indication that the conserved pentapeptide sequence regulates SERCA1a function.
by interacting with the membranes. With appropriate control experiments it is possible to attribute the peaks in the spectrum to ligands bound to specific receptors. Here $^{13}$C CP-MAS NMR was used to examine how the $^{13}$C-labeled peptide [α-13C-Tyr]$^{3}$NAC-RSYQY interacts with SERCA1a when it is reconstituted alone into DOPC membranes or together with SLN. The experiments were carried out at 4 °C so as to minimize the dynamics of NAC-RSYQY within the membrane sample and thus provide favorable conditions for observing signals for the bound peptide.

First it was necessary to establish that the labeled peptide could be detected when bound to SERCA1a. The $^{13}$C spectrum of DOPC membranes containing SERCA1a and 100 μM [α-13C-Tyr]$^{3}$NAC-RSYQY exhibits a sharp peak from the peptide at −56 ppm, superimposed on a broad envelope of peaks from $^{13}$C at natural abundance in the lipids and protein. The peak assigned to the peptide indicated that the mobility of [α-13C-Tyr]$^{3}$NAC-RSYQY is restrained in the presence of the SERCA1a membranes (Fig. 6a, top). A second spectrum was obtained from an identical sample containing 200 μM unlabelled NAC-RSYQY to displace the labeled peptide from specific binding sites but leaving nonspecifically bound peptide (not shown). A difference spectrum was then obtained by subtraction of the second spectrum from the first (Fig. 6a, bottom). The remaining peak at 56 ppm in the difference spectrum corresponds to [α-13C-Tyr]$^{3}$NAC-RSYQY that is displaced from its binding site(s) in the membrane by addition of excess unlabeled peptide, and it may represent a peptide that is in direct contact with SERCA1a.

To confirm the origin of the peak in the difference spectrum, spectra were next obtained from a SERCA1a membrane sample in the presence of the peptide [α-13C-Ala]$^{3}$NAC-RSAQY, which does not inhibit SERCA1a and appears to have little or no affinity for the NAC-RSYQY-binding site as will be shown later in Fig. 7. Neither the initial spectrum nor the difference spectrum show peaks consistent with motionaly restrained peptide (Fig. 6b). Similarly, no evidence for restrained peptide was observed in spectra of DOPC membranes containing SLN and [α-13C-Tyr]$^{3}$NAC-RSYQY but not SERCA1a (Fig. 6c). It thus appears that peaks for the motionaly restrained peptide are observed only if the labeled peptide interacts with SERCA1. Further evidence supporting this conclusion was provided by NMR spectra of [α-13C-Tyr]$^{3}$NAC-RSYQY in Na$^{+}$/K$^{+}$/ATPase membranes (at a similar protein concentration to the SERCA1a membranes). The peptide does not inhibit Na$^{+}$/K$^{+}$/ATPase (Fig. 4a), and correspondingly, no peaks for the peptide were observed in the spectra (Fig. 6d). It is therefore reasonable to conclude that the sharp peak for [α-13C-Tyr]$^{3}$NAC-RSYQY observed in Fig. 6a originates from the peptide interacting directly with SERCA1a.

A $^{13}$C NMR spectrum was next obtained to examine the effect of SLN on the interaction between SERCA1a and NAC-RSYQY. The peptide [α-13C-Tyr]$^{3}$NAC-RSYQY was added to a DOPC membrane sample containing SERCA1a and a 20-fold higher concentration of SLN (Fig. 6e). The sample was prepared under identical conditions to the sample used in Fig. 6a, and it differed only in the presence of SLN. At such a high SLN concentration, it is expected that most of the SERCA1a in the mem-

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**FIGURE 4. Analysis of the effects of the SLN peptide fragment NAC-RSYQY on the rate of ATP hydrolysis by SERCA1a in purified SR membranes.** A dose-response curve of the ATPase activity (at 1.5 μM Ca$^{2+}$ and 3 μg of protein) of SERCA1a in the presence of different concentrations of NAC-RSYQY (squares) reveals an IC$_{50}$ for the peptide of −200 μM (a). NAC-RSYQY has no effect on Na$^{+}$/K$^{+}$/ATPase activity (130 mM Na$^{+}$, 20 mM K$^{+}$, 4 mM Mg$^{2+}$, and 2 mM ATP) over the same peptide concentration range (circles). Data are expressed as a percentage of hydrolytic activity in the absence of peptide (4.5 μmol/mg per min for SERCA1a and 28.3 μmol/mg per min for Na$^{+}$/K$^{+}$/ATPase). The rates of ATP hydrolysis by purified SERCA1a were measured as a function of calcium concentrations of up to 2.5 μM (b). The rates of hydrolysis by SERCA1 alone (squares) and after the addition of 100 μM NAC-RSYQY (circles) indicate that the peptide lowers $V_{\text{max}}$ for ATP hydrolysis to −70% of the rate in the absence of peptide.
FIGURE 5. A series of experiments on SR vesicles to determine the sidedness of SERCA1a inhibition by NAc-RSYQY. First, experiments were performed on freshly prepared SR vesicles before treatment with detergent (a). Electron micrographs confirm that the vesicles were predominantly sealed (left) so that, in the presence of external ATP, SERCA1a molecules oriented with the cytoplasmic face toward the vesicle exterior transports calcium into the vesicle (middle schematic). The addition of NAc-RSYQY to the vesicles to a final peptide concentration of 200 μM has no effect on the rate of ATP-dependent Ca\(^{2+}\) uptake at calcium concentrations of up to 150 mM (right), suggesting that the peptide does not act at the cytoplasmic face of the enzyme. Next, the vesicles were disrupted by the addition of 0.1% C\(_{12}\)E\(_{8}\) (b). Electron micrographs confirm that the vesicles were fractured (left), and addition of 200 μM NAc-RSYQY to the fractured vesicles allowed equal access of the peptide to both faces of SERCA1a (middle schematic). The broken vesicles in the absence and presence of the peptide can no longer accumulate Ca\(^{2+}\), as expected (right). The detergent was then removed by absorption to polystyrene beads (c). Electron micrographs confirm that the vesicles had reformed (left) leaving NAc-RSYQY present on the inside and outside the reformed vesicles (middle schematic). Ca\(^{2+}\) uptake is partially restored (right), but NAc-RSYQY reduces the rate by ~40% (circles) relative to the rate of uptake by control reformed vesicles prepared without exposure to the peptide at any stage (triangles). Addition of 200 μM NAc-RSYQY to the control, reformed vesicles (squares) has no effect on the calcium uptake rate, thereby eliminating the possibility that exposure of SERCA1a to detergent induced structural rearrangements of the enzyme revealing a binding site for the peptide on the outward-facing cytoplasmic face. All calcium uptake rates are expressed as a percentage of the maximal uptake rate by untreated SR vesicles in the absence of peptide (0.3 μmol of Ca\(^{2+}\)/mg per min).
Structure/Function Studies of Sarcolipin-SERCA Interactions

![Diagram of SERCA1a and SLN](image)

- **a** SERCA1a
- **b** SERCA1a
- **c** SLN/DOPC
- **d** Na⁺/K⁺-ATPase
- **e** SERCA1a + SLN

**FIGURE 6.** ¹³C CP-MAS NMR experiments to observe NAc-RSYQY binding to SERCA1a in the presence and absence of SLN at 4 °C. The spectra were obtained from membrane samples comprising either DOPC/SERCA1a in a 160:1 molar ratio (a and b), DOPC/SLN in a 20:1 molar ratio (c), a membranous Na⁺/K⁺-ATPase preparation (~5 mg of protein) (d), and DOPC/SLN/SERCA1a in a 160:20:1 molar ratio (e). The top spectra were obtained from samples containing either 100 μM [¹³C-Ca³⁺]NAc-RSYQY (a, c, d, and e) or [¹³C-Ca³⁺]NAc-RSAQY (b). From the top spectra were subtracted fresh spectra obtained from the same samples after adding 200 μM unlabeled NAc-RSYQY (a, c, d, and e) or unlabeled NAc-RSAQY (b), to generate the difference spectra (Δ). Any labeled peptide that is displaced by unlabeled peptide is observed as a peak in the difference spectrum. Peaks from [¹³C-Ca³⁺]NAc-RSYQY and from the naturally abundant ¹³C present in the lipids and protein are highlighted. The sample spinning rate was 4 kHz.

brane is complexed with SLN. In contrast to Fig. 6a, neither the initial spectrum nor the difference spectrum showed peaks corresponding to [α⁻¹³C-Tyr⁴⁺]NAc-RSYQY, indicating that full-length SLN prevents the peptide from being restrained by SERCA1a. This observation is consistent with SLN and [α⁻¹³C-Tyr⁴⁺]NAc-RSYQY sharing a common binding site in SERCA1a, located at the luminal face as established above.

**Sequence Specificity of SERCA1 Inhibition**—The importance of the two tyrosine residues of NAc-RSYQY for inhibition of SERCA1a was examined by measuring calcium-dependent ATPase activity in the presence of the nonaromatic peptide analogue NAc-RSAQA and 9 other analogues in which the original five residues of NAc-RSYQY were either replaced with conservative or nonconservative substitutions or were truncated from the N-terminal end. At a concentration of 100 μM (approximately the IC₅₀ of NAc-RSYQY), none of the peptides had a significant effect on the calcium affinity of SERCA1a, as the midpoint of the curves fell at approximately the same calcium concentration for each of the analogues (Fig. 7a). The effect of the peptides on Vₘₐₓ was also determined (Fig. 7b). NAc-RSYQY reduced Vₘₐₓ by the largest amount, but replacement of the tyrosine residues with phenylalanine also gave a slight inhibitory effect, reducing Vₘₐₓ by ~15%. Replacement of either one of the tyrosines with alanine gave no effect on Vₘₐₓ, suggesting that aromatic or bulky hydrophobic side groups at the two substituted residues are essential for an inhibitory effect. Interestingly, [lysine/arginine, glutamic acid/arginine, asparagine/glutamine, or glutamic acid/glutamine] substitutions also reduced the inhibitory potentials of the peptides relative to that of NAc-RSYQY, as did truncation of the first two N-terminal residues. Hence, these experiments reveal that the inhibitory function of NAc-RSYQY is remarkably sensitive to changes to any of the five residues in the native pentapeptide sequence and not only the tyrosine groups.

**Combined Effects of SLN and PLB Peptides**—Earlier studies by MacLennan and co-workers (9) revealed that SLN associates with both PLB and SERCA to form a superinhibitory ternary complex. It was proposed that the enhanced inhibition of calcium transport could arise because the transmembrane domains of SLN and PLB fit into the cleft formed by transmembrane helices M2, M4, M6, and M9 of the enzyme more tightly than does either protein alone. It is not clear, however, if the combined transmembrane regions of SLN and PLB simply act as an efficient anchor by lowering the dissociation rate of the complex or if they are also directly responsible for superinhibition of SERCA from within the lipid bilayer. The cytoplasmic domain of PLB also contributes toward the regulation of activity by making contact with residues in the nucleotide binding region (37). We therefore explored the possibility that the enhanced inhibitory effect of the PLB-SLN complex could originate at sites outside the membrane, from the concomitant regulation of SERCA by the cytoplasmic face of PLB and by SLN at the luminal face.

A water-soluble peptide PLB-(1–23), consisting of the first 23 N-terminal residues representing most of the cytoplasmic domains of PLB, inhibits calcium uptake by SR vesicles with an IC₅₀ in the 10⁻⁵ M range (Fig. 8a). The peptide P-PLB-(1–23), phosphorylated at Ser₁⁶, also inhibited calcium uptake, although slightly less effectively than PLB-(1–23). The peptides must therefore inhibit SERCA1a at the cytoplasmic face, consistent with a physiologically relevant effect on transport activity. The effect of PLB-(1–23) on the ATPase activity of SERCA1a purified in nonvesicular membranes was examined at different concentrations of NAc-RSYQY. In this preparation...
both faces of the enzyme are accessible to the peptides and nucleotide. At NAc-RSYQY concentrations in the $10^{-4}\text{ M}$ range the addition of PLB-(1–23) enhanced inhibition by up to 40% relative to NAc-RSYQY alone (Fig. 8b). These results are therefore consistent with, but not conclusive of, PLB and SLN having a cumulative effect on SERCA1a activity as a result of interactions with sites on opposite faces of the enzyme and independently of their transmembrane regions. Further experiments are underway to provide more definitive proof that PLB and SLN cooperatively inhibit SERCA1a.

**DISCUSSION**

It is now clear that SLN, like PLB, regulates calcium cycling through a direct inhibitory association with SERCA enzymes in skeletal muscle and cardiac myocytes. Co-expression of SLN with SERCA1a or SERCA2a in HEK-293 cells reduces the apparent affinity of the enzyme for calcium and also affects the maximum rate of calcium transport. Early studies by MacLennan and co-workers (6) reported that SLN stimulated $V_{\text{max}}$ for calcium transport, but more recent work by the same group and others (7–9) suggest that the $V_{\text{max}}$ for both SERCA1a and SERCA2a is lowered slightly ($\leq 10\%$) by SLN and to a much greater extent when SLN combines with PLB. Our results agree with recent studies and show that SLN lowers both $V_{\text{max}}$ and the apparent affinity of SERCA1a for calcium (Fig. 1a).

The C-terminal sequence RSYQY of SLN has been highlighted as being important for targeting the protein to the SR membrane, and there are also clues suggesting that the same sequence plays a role in the interaction between SLN and SERCA, because null-tyrosine mutants of FLAG-tagged SLN co-expressed with SERCA in HEK-293 cells effect the rate of Ca$^{2+}$ uptake by SR microsomes (6). To date, however, there has been no explicit evidence confirming that the side groups of Tyr$^{29}$ and Tyr$^{31}$ associate specifically with SERCA in lipid bilayers or that they are involved directly in the regulation of enzyme activity and calcium transport. Here, with solid-state NMR experiments on SLN in situ within lipid bilayers containing SERCA1a, it is revealed that the aromatic rings of Tyr$^{29}$ and Tyr$^{31}$ of SLN participate in a detectable interaction with SERCA1a. This work has also shown that the five C-terminal amino acids of SLN associate with the luminal face of the enzyme in isolation from the transmembrane domain and lower the maximal rates of ATP hydrolysis and Ca$^{2+}$ uptake but not the affinity of SERCA1a for calcium. Full-length SLN affects both the calcium affinity of SERCA1a and $V_{\text{max}}$ of ATP hydrolysis (Fig. 2a), and SLN and NAc-RSYQY appear to share a common binding site for SERCA1a (Fig. 6). It is therefore reasonable to propose that the conserved C-terminal residues of full-length SLN contribute to the depression of $V_{\text{max}}$ and the transmembrane domain is responsible for lowering calcium affinity for the enzyme. It was found that by substituting alanine for the two tyrosines in NAc-RSYQY the peptide has no effect on the activity of SERCA1a, suggesting that two aromatic residues are crit-

![Image](https://example.com/image.png)

**FIGURE 7. Analysis of the effects of short analogues of NAc-RSYQY on the rate of ATP hydrolysis by SERCA1 in purified SR membranes.** The rates of ATP hydrolysis by purified SERCA1a are shown as a function of calcium concentrations of up to 2.5 $\mu\text{M}$ in the presence of NAc-RSAQA and 9 other analogues at a peptide concentration of 100 $\mu\text{M}$ (a). All activities are normalized to $V_{\text{max}}$ for each curve. None of the peptides had a significant effect upon the activity of SERCA1a in the presence of NAc-RSAQA and 9 other analogues at peptide concentrations of 100 $\mu\text{M}$ indicate that substitution or truncation of the amino acids of NAc-RSYQY results in a diminished inhibitory effect (b). Rates are expressed as percentage $V_{\text{max}}$ for SERCA1 alone (4.5 $\mu$moles/mg per min) at the Ca$^{2+}$ concentration of 2.5 $\mu\text{M}$ used in all the experiments here.

![Image](https://example.com/image.png)

**FIGURE 8. Analysis of the effects of the PLB peptide fragments PLB-(1–23) and P-PLB-(1–23) on SERCA1a activity.** Both peptides inhibited calcium uptake by SR vesicles over a peptide concentration range of 1–100 $\mu\text{M}$ and a calcium concentration of 1 $\mu\text{M}$ Ca$^{2+}$ (a). A dose-response curve of the ATPase activity (at 1 $\mu\text{M}$ Ca$^{2+}$ and 3 $\mu$g of protein) of purified SERCA1a in the presence of different concentrations of NAc-RSYQY and in the absence or presence of 10 $\mu\text{M}$ PLB-(1–23) reveals that the presence of both peptides enhances inhibition of hydrolytic activity by up to 40% (relative to inhibition by NAc-RSYQY alone) (b). Data are expressed as a percentage of hydrolytic activity in the absence of NAc-RSYQY (4.5 $\mu$mol/mg per min).
ical for inhibition of SERCA1a. However, the poor inhibitory activities of mutated and truncated variants of NAc-RSYQY indicate that all five native residues are essential for inhibition. The positively charged residue Arg27 may be important in stabilizing the interaction of RSYQY with Ca\(^{2+}\)-ATPase, possibly through salt bridge formation, and the two tyrosines could participate in π-π or π-cation interactions with residues at the luminal face of SERCA1a.

These results raise the question of how the C-terminal amino acids in full-length SLN are able to lower the rate of ATPase-dependent calcium transport by SERCA1a. The relatively high IC\(_{50}\) of NAc-RSYQY indicates that binding to SERCA1a is rather weak and is consistent with the recognition site being situated at a luminal surface loop and not within the transmembrane helices where the inhibitor thapsigargin binds (38). In this respect, the peptide, and its corresponding sequence in full-length SLN, may act similarly to luminally active reversible inhibitors of gastric H\(^{+}/K^{+}\)-ATPase, which interact with loop regions and stabilize enzyme intermediates in the catalytic cycle. Molecular modeling suggests that transmembrane helices M2, M4, M6, and M9 of SERCA1a form a groove into which fits the transmembrane helix of SLN, allowing interactions between the aromatic residues of the SLN C-terminal domain and the loop region between M1 and M2 of SERCA (9). In this arrangement, Tyr29 can make contact with Phe73, Trp77, and Phe88 of SERCA1a, and Tyr31 can interact with Phe88 and Ile86. Detailed structure determination of SERCA1a locked in conformations analogous to the E\(_1\)E\(_{1}\)E\(_{2}\)Ca\(^{2+}\), E\(_2\)-ADP-P\(_{i}\)E\(_{2}\)P, and E\(_{2}\) intermediate states in the catalytic cycle reveal gross structural changes in the cytoplasmic nucleotide binding (N) and actuator (A) domains, mechanically coordinated by the phosphorylation (P) domain (38, 39). ATP binding to the N domain is accompanied by a large scale movement in the N domain and a 30° rotation of the A domain to bring the two domains close enough together to allow the γ-phosphate of ATP and Mg\(^{2+}\) to interact with the phosphorylation site in the P domain. Large structural changes also occur upon dissociation of bound calcium, with the A domain rotating horizontally by 110°. These structural changes in the cytoplasmic domains are transmitted to the transmembrane helices M1–M6, which undergo concomitant rearrangements during the catalytic cycle. In the E\(_1\)E\(_{1}\)E\(_{2}\)Ca\(^{2+}\) state, the M1 helix is deeply embedded in the lipid bilayer and stabilized by bound calcium, but ATP binding pulls the M1 helix up through the membrane and bends it at the cytoplasmic end (38). Such a movement will affect the positions of the M1–M2 loop residues to which the RSYQY motif is predicted to bind; expressed another way, binding of the RSYQY motif to the M1/M2 loop residues may lock the enzyme in one of its conformational intermediates, thereby preventing the domain reorientations necessary for ATP hydrolysis or the dissociation of calcium, for example.

In summary, this work demonstrates how a combination of solid-state NMR and functional measurements of protein-protein interactions can be used to reveal important new information relevant to how calcium cycling is regulated in muscle cells. We have focused on SERCA1a from skeletal muscle, but previous studies of the functional regulation of SERCA enzymes show that the effects of SLN on the skeletal and cardiac isoforms are broadly similar (8). The functional effects of regulatory proteins PLB and SLN on Ca\(^{2+}\)-ATPase are comparable for both SERCA1 and SERCA2a isoforms, and the use of SERCA1 as an experimental model for cardiac isofrom SERCA2a has been well established across a range of reconstitution (40–42) and co-expression (8, 43) studies. The biochemical properties of SERCA1 and SERCA2a are virtually identical to each other in terms of Ca\(^{2+}\)-dependent activity, and an identity reduction in their affinity for Ca\(^{2+}\) is seen when the two isoforms are co-expressed with PLB (43, 44). Hence our findings with SERCA1 could be relevant to the regulation of SERCA2a by SLN in cardiac atria. For instance, overexpression of SLN impairs the contractility of cardiac myocytes (10, 11), and SLN mRNA expression is down-regulated in chronic atrial fibrillation (12). It has been suggested that the regional differences in the expression levels of SLN and PLB within the heart may differentiate the contractile parameters of the atria from those of the ventricles (13). The pathogenesis of cardiac disorders can be traced to perturbations in calcium cycling, suggesting that the alteration of SERCA2a function by SLN may contribute to the development of disease and progression to heart failure. With emerging information about the importance of SLN in normal and diseased cardiac tissue, locating which amino acid residues of SLN interact with SERCA enzymes and regulate calcium transport is a valuable step toward identifying target sites for therapeutic intervention in the treatment of cardiac disorders and provide motivation for further examination of the regulatory effect of the RSYQY sequence in full-length SLN, both in vitro and in vivo.

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