Targeted Overexpression of Sarcolipin in the Mouse Heart Decreases Sarcoplasmic Reticulum Calcium Transport and Cardiac Contractility*

The role of sarcolipin (SLN) in cardiac physiology was critically evaluated by generating a transgenic (TG) mouse model in which the SLN to sarco(endoplasmic)reticulum (SR) Ca\textsuperscript{2+} ATPase (SERCA) ratio was increased in the ventricle. Overexpression of SLN decreases SR calcium transport function and results in decreased calcium transient amplitude and rate of relaxation. SLN TG hearts exhibit a significant decrease in rates of contraction and relaxation when assessed by ex vivo work-performing heart preparations. Similar results were also observed with muscle preparations and myocytes from SLN TG ventricles. Interestingly, the inhibitory effect of SLN was partially relied upon high dose of isoproterenol treatment and stimulation at high frequency. Biochemical analyses show that an increase in SLN level does not affect PLB levels, monomer to pentamer ratio, or its phosphorylation status. No compensatory changes were seen in the expression of other calcium-handling proteins. These studies suggest that the SLN effect on SERCA pump is direct and is not mediated through increased monomerization of PLB or a change in PLB phosphorylation status. We conclude that SLN is a novel regulator of SERCA pump activity, and its inhibitory effect can be reversed by β-adrenergic agonists.

The sarco(endoplasmic)reticulum (SR) Ca\textsuperscript{2+} ATPase (SERCA) plays a dominant role in transporting Ca\textsuperscript{2+} into the SR during the contraction-relaxation cycle of the heart. The rate and amount of Ca\textsuperscript{2+} transported into the SR determines both the rate of muscle relaxation and the SR Ca\textsuperscript{2+} load available for the next cycle of contraction (1–4). It is well established that SERCA function is regulated by phospholamban (PLB), whose inhibitory effect is reversed by phosphorylation by protein kinase A and the calcium/calmodulin-dependent protein kinase (CAMKII) during adrenergic activation (5–7). Recent studies have shown that in addition to PLB, sarcolipin (SLN) could also play an important role in the regulation of SERCA pump activity (8–12).

SLN is a 31-amino acid protein expressed in both cardiac and skeletal muscle (11, 13–15). We have recently demonstrated that SLN is localized in the cardiac SR membrane, and its distribution pattern is similar to SERCA2a and PLB (11). SLN mRNA is differentially expressed in small as opposed to larger mammals. In rodents, SLN mRNA is abundant in the atria with very low levels in the ventricle and skeletal muscles (11, 14, 15). In contrast, in larger mammals including humans, SLN mRNA is abundant in fast-twitch skeletal muscle compared with atria and ventricle (13). SLN expression is developmentally regulated (11), and its expression levels are modified under certain pathological conditions of the muscle (16, 17). Decreased expression of SLN mRNA has been shown in the atria of patients with atrial fibrillation (16). A recent study also showed that SLN mRNA was up-regulated ~50-fold in the hypertrophied ventricles of Nkx2–5 null mice (17).

Structural similarities between SLN and PLB indicate that they are homologous proteins and may functionally substitute for each other (9, 10, 18, 19). Recent studies carried out in HEK cells showed that SLN could inhibit the SERCA pump activity (8, 18). Co-expression of SLN with either SERCA1a or SERCA2a decreases the apparent Ca\textsuperscript{2+} affinity of the SERCA pump. Furthermore, when SLN and PLB are co-expressed, SLN was shown to inhibit the polymerization of PLB, resulting in more monomers and super-inhibition of the SERCA pump (8, 20). Using adenoviral gene transfer into cardiac myocytes, we recently demonstrated that overexpression of SLN resulted in decreased myocyte contractility and calcium handling. However, overexpression of SLN did not alter the PLB pentamer/monomer ratio in cardiac myocytes (11).

Based on our adenoviral gene transfer studies in cardiac myocytes (11), we hypothesized that SLN can directly modulate SERCA pump activity and affect cardiac contractility. To test this hypothesis, we specifically altered the SLN to SERCA ratio in the ventricle by overexpressing SLN using the α-MHC gene promoter. Results presented in this study suggest that SLN directly inhibits the SERCA pump activity, and its inhibitory effect can be reversed upon adrenergic stimulation and increased frequency.

**EXPERIMENTAL PROCEDURES**

All experiments were performed in accordance with National Institutes of Health guidelines and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

**Generation of Transgenic Mice—N-terminal FLAG-tagged mouse SLN cDNA (11) was amplified by PCR and ligated into the Sall and HindIII sites downstream of the 5.5-kb mouse α-MHC promoter and upstream of the poly(A) signal sequence from the human growth hormone. The complete recombinant construct was excised from the plas-
mid backbone by NotI restriction digestion and gel-purified. To generate transgenic founder mice, DNA samples were microinjected into the pronuclei of C57BL/6 murine embryos at the core facility for transgenics, University of Cincinnati.

Mice carrying the transgene were identified by PCR analysis using primers specific for α-MHC (5′-GCCCAACACAGGAAATGACAGA-3′) and the antisense primer specific for the 3′-end of SLN cDNA (5′-TCAGATTTGGTAGGACCTA-3′). The copy number of the transgene was identified by Southern blot analysis of DNA samples from TG mice as described earlier (23).

**Determination of SERCA/SLN Ratio by RT-PCR**—Total RNA was isolated from ventricle or atria (pooled from two mice) using the ULTRASPEC-II RNA Isolation System (Biotecx Labs., Houston, TX). RT-PCR analysis was done using 1 μg of total RNA from ventricle or atria as described earlier (22). Following oligo(dT)-primed first-strand cDNA synthesis, 1-μl portions of the first-strand cDNA mixture were subjected to PCR using primers specific for mouse SERCA (forward, 5′-CTGGGAGACCTCTGGTTGT-3′ and reverse, 5′-CAGAGACAGATGGTTGGCATA-3′), mouse SLN (forward, 5′-GACATGGTGCCATGGT-3′ and reverse, 5′-ACTAAGGACTGGACAGAG-3′), NF-SLN (FLAG forward, 5′-CTACAGGACGACAGTG-AACA and human growth hormone poly(A) reverse, 5′-AGGTTTGTCTTGGCATGT-3′), mouse DHPR (forward, 5′-CCCATCACCATCTTCCAGA-3′ and reverse, 5′-TTGCTCATACAGGAAATGAGC-3′). PCR was adjusted to obtain equal amounts of SERCA2a, and the number of cycles was chosen to fall within the exponential phase of amplification. Total SLN mRNA levels were calculated by adding endogenous and NF-SLN mRNA levels. The PCR protocols were as follows: 94 °C for 30 s, 55 °C for 30 s, and 75 °C for 60 s (35 cycles) with a 72 °C extension for 7 min.

**Western Blot Analysis**—Cardiac homogenate was prepared from transgenic and non-transgenic ventricles, and Western blot analysis was carried out as described earlier (11, 23). Briefly, equal amounts of total homogenates from SLN TG and NTG ventricles were separated on: 5% (for RyR, NCX, and PMCA) 8% (for SERCA and CSQ), 10% (for DHPRα2 and triadin), and 14% (for PLB and NF-SLN) SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were immunoprobed with the following primary antibodies: anti-rabbit SERCA2a, anti-rabbit PLB, anti-rabbit CSQ (ABR), anti-mouse DHPRα2, anti-rabbit triadin, anti-mouse PMCA (ABR), anti-mouse NCX (Swant, Switzerland), anti-rabbit S16 or T17 PLB antibody (Cyclacel, Dundee, UK). Protein loading was normalized to Coomassie Blue staining and α-actin levels. Signals were detected by SuperSignal WestDura substrate (Pierce) and quantitated by densitometry.

To determine β-adrenergic agonist-mediated PLB phosphorylation, SLN TG hearts were perfused with isoproterenol in an isolated work-performing heart setup as described below. One set of hearts was freeze-clamped after 30 min without isoproterenol, and the other set was treated with isoproterenol (1 μM) for 5 min after 25 min of perfusion. PLB phosphorylation was estimated by Western blotting analyses.

**Calcium Uptake Assay**—Ventricles from TG and NTG mice were used for calcium uptake assays as described earlier (23, 24). Briefly, ventricular tissue was homogenized in 8 volumes of protein extraction buffer (in mmol/liter, 50 KPi, 10 NaF, 1 EDTA, 300 sucrose, 0.5 dithiothreitol, and 0.3 phenylmethylsulfonyl fluoride), and calcium uptake was measured by the Millipore filtration technique. Ventricular homogenates (150 μg) from NTG and SLN TG animals were incubated at 37 °C in a 1.5 ml of calcium uptake medium (in mmol/liter, 40 imidazole, pH 7.0, 100 KCl, 5 MgCl2, 5 Na2ATP, 5 potassium oxalate, and 0.5 EGTA) and various concentrations of CaCl2 to yield 0.03–3 μmol/liter free Ca2+ (containing 1 μCi/μmol 45Ca2+). To obtain the maximal stimulation of SR Ca2+ uptake, 1 μM ruthenium red was added immediately prior to the addition of the substrates to begin the calcium uptake. The reaction was initiated by the addition of 5 μM ATP and terminated at 1 min by filtration. The rate of calcium uptake and the Ca2+ concentration required for half-maximal velocity of Ca2+ uptake (EC50) were determined by non-linear curve fitting analysis using GraphPad PRISM 4.0 software.

**Isolated Work-performing Heart Preparations**—Work-performing heart preparations were performed as described previously (23, 25). Mice were anesthetized via intraperitoneal injection with 100 mg/kg sodium nembutal and 1.5 units of heparin to prevent intracoronary microthrombi. The aorta was cannulated, and retrograde perfusion (Langendorff mode) was carried out at a constant perfusion pressure of 50 mmHg with Krebs-Henseleit buffer containing (in mmol/liter) 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 0.5 Na-EDTA, 25 NaHCO3, 1.2 KH2PO4, and 11 glucose. All perfusion buffers were equilibrated with 95% O2 plus 5% CO2, yielding a pH of 7.4. A water-filled catheter (P-50) was inserted through mitral valve into the left ventricle. After a short period of stabilization on retrograde perfusion, the pulmonary vein was cannulated, and perfusion of the heart was switched from retrograde to antegrade. A 20-gauge cannula was tied into the left pulmonary vein to accommodate regulation on recording of venous return. Antegrade work-performing perfusion was initiated at a workload of 250 mmHg/m, which was achieved using a custom micrometer-controlled venous return of 5 ml/min and an aortic pressure of 50 mmHg. After establishment of the base line, responses to infusion of β-adrenergic receptor agonist, isoproterenol, by a microperfusion pump (Master flex) were measured with varying concentrations for 2 min. The signals were digitized, and the following indices of cardiac performance were measured off-line using Biobench software (National Instruments, Inc): left ventricular systolic pressure, end diastolic pressure, diastolic pressure, end systolic pressure, left ventricular systolic pressure, end diastolic pressure, diastolic pressure, the minimum (−dp/dt) and maximum (+dp/dt) derivatives of left ventricular pressure, time to peak systolic pressure (TPP), and time to reach 50% of relaxation (TR1/2). TPP and TR1/2 were normalized with respect to peak left ventricular pressure because they are dependent upon extent of pressure development. Force-frequency relationship was carried out to assess frequency-dependent contractile reserve. For these experiments, hearts were paced with frequencies from 4 to 12 Hz, and +dp/dt and −dp/dt were determined at multiple intervals.

**Preparation of Muscle Fibers and Experimental Setup**—Small, unbranched trabeculae or the smallest of the RV papillary muscles were dissected from the right ventricle as previously described (26). The dimensions of the preparations were 221 ± 21 μm, 174 ± 18, and 1401 ± 78 (width × thickness × length in μm, n = 15) and are not different between the two groups. Using the dissection microscope, muscles were mounted between a platinum-iridium basket-shaped extension of a force transducer (KG7, Scientific Instruments GmbH, Heidelberg, Germany) and a hook (valve end) connected to a micromanipulator. Muscles were superfused with Krebs-Henseleit buffer ([Ca2+]i, 1.5 mmol/liter) at 37 °C and stimulated at baseline (4 Hz). Muscles were stretched to a length where a small increase in length resulted in nearly equal increases in resting tension and active developed tension (26). This length was selected to be comparable to a length close to the end of diastole.

After stabilization, contractile parameters were recorded at 4 different muscle lengths between slack and optimal length, stimulated at rates between 4 and 14 Hz in a second protocol, and finally the response to a concentration-response curve of isoproterenol was
amplification as described under "Experimental Procedures." The heart was then perfused with blenzyme solution and filtered through a 200 micron nylon mesh. After triturating with a large bore pipette, the remaining pieces were post-digested with additional blenzyme solution and filtered through a 200 micron nylon mesh. The tissue was then dissected in a Petri dish with fresh blenzyme solution, removing atria, and chopping into fine pieces. After triturating with a large bore pipette, the remaining pieces were post-digested with additional blenzyme solution and filtered through a 200 micron nylon mesh. All myocyte suspensions were spun for 20 sec at 1000 rpm, supernatant decanted, and resuspended in modified MEM to which 0.1% bovine serum albumin and 50 μM Ca²⁺ were added. After gravity-settled, cells were resuspended in MEM containing 200 μM Ca²⁺.

Myocytes were loaded at 22 °C with Fluo-4 AM (10 μM, Molecular Probes, Eugene, OR) for 30 min for intracellular de-esterification. The illumination field was restricted to a small spot to get emission from a single cell. Data were expressed as F/F⁰, where F is the fluorescence intensity and F⁰ is the intensity at rest. Simultaneous measurement of shortening was also performed using an edge detection system (Crescent Electronics, Sandy, UT). Data were expressed as % of resting cell length. Myocytes were field-stimulated via platinum electrodes connected to a Grass S48 stimulator, with Ca²⁺ transients and myocyte shortening simultaneously measured (29). Force-frequency relationship (0.2, 0.5, 1, and 2 Hz) and isoproterenol dose-response curves were generated.

Statistics—Results are expressed as mean ± S.E. Statistical significance was estimated by a paired Student's t test. A value of p < 0.05 was considered statistically significant.

RESULTS

Generation of SLN TG Mice—To determine how SLN regulates cardiac function and SR calcium transport, we overexpressed FLAG-tagged mouse SLN cDNA under the control of the α-MHC promoter (Fig. 1A). The TG mice were generated in a CB56 background and the progeny screened for germline transmission of the transgene. PCR analysis indicated that 4 of 52 of initial F₀ mice carried the transgene. Out of 4 founders, only 2 of them (line 20 and line 26) were fertile and produced progeny. Southern blot analysis revealed mouse lines 20 and 26 carried 4 founders, only 2 of them (line 20 and line 26) were fertile and produced progeny. Further our data suggest that NF-SLN expression in various muscle tissues including cardiac, skeletal, and vascular smooth muscle indicates that NF-SLN expression is restricted to heart and not detectable in other tissues analyzed (Fig. 1B).
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FIGURE 2. Quantification of SR calcium-handling proteins and sarcolemmal calcium transporters, NCX and PMCA. Different SDS-PAGE gel concentrations (5% for RyR, NCX, and PMCA, 8% for SERCA and CSQ, 10% for DHPRα2 and triadin, and 14% for PLB) were used to resolve total ventricular homogenates from NTG and TG mice and immunoprobed with specific antibodies.

FIGURE 3. Basal phosphorylation of PLB in SLN TG ventricles. Total homogenates were unboiled and analyzed for PLB monomers (PLB1–16) and pentamers (PLB1–17) by Western blot analysis. To detect PLB monomer levels, higher amounts of protein (10 μg and 20 μg) were loaded. To determine the basal phosphorylation of PLB, snap-frozen samples were processed for protein extraction and immunoprobed with Ser16- or Thr17-specific PLB antibodies. Data are representative of three independent experiments.

Expression Levels of SR Calcium-handling Proteins Are Unchanged in Transgenic Mice—Quantitative Western blot analysis was carried out to determine if increased SLN expression affected the levels of SERCA and PLB in the TG ventricle. Our results show that the expression levels of SERCA2a, PLB, and CSQ were unchanged in the TG ventricle (Fig. 2A) indicating that expression of these proteins was not affected by SLN overexpression. We also quantitated the expression levels of ryanodine receptor (RyR), L-type calcium channel subunit-dihydropyridine receptor α2 (DHPRα2), and triadin to determine changes in Ca2+ release and entry mechanisms. As shown in Fig. 2B, SLN overexpression did not affect the RyR, DHPRα2, or triadin levels. To determine whether SLN inhibition of the SERCA pump is associated with compensatory changes in the expression of other plasma membrane calcium extrusion systems, we quantitated the sodium-calcium exchanger (NCX) and plasma membrane calcium ATPase (PMCA) levels. Results in Fig. 2B indicate that SLN overexpression did not alter the expression of NCX and PMCA protein levels.

Basal Phosphorylation of PLB Is Not Affected by SLN Overexpression—In a recent study, Asahi et al. (12) reported that cardiac-specific overexpression of SLN resulted in decreased basal phosphorylation of PLB and an increase in the monomer to pentamer ratio. To test whether overexpression of SLN resulted in monomerization of PLB and decreased phosphorylation, Western blot analysis was carried out using appropriate antibodies. Our results (Fig. 3) clearly show that the monomer to pentamer ratio was not altered in the TG ventricle. Further, the basal phosphorylation of PLB at serine 16 (Ser16) and threonine 17 (Thr17) was not different between the TG and NTG ventricles (Fig. 3). These results contradict a previous report (12) and show that SLN overexpression did not alter either PLB basal phosphorylation or the monomer to pentamer ratio.

SLN Overexpression Decreases the Apparent Ca2+ Affinity and Rate of Ca2+ Uptake—To determine the effect of the increased SLN to SERCA ratio on SR calcium transport, the rate of calcium dependence of calcium uptake was measured in total ventricular homogenates from SLN TG and NTG ventricles. Results show that there is a significant rightward shift in the sigmoid curve measuring calcium dependence of calcium uptake in TG mice indicating a reduced Ca2+ affinity in SLN TG ventricles (Fig. 4). The EC50 value for Ca2+ increased significantly in the TG ventricle (NTG, 163.8 ± 12.64 nM versus SLN TG, 209.3 ± 20.62 nM; n = 4; p < 0.05) when compared with the NTG ventricle. However, the maximum velocity (Vmax) of Ca2+ uptake was not significantly different between NTG and TG ventricles (NTG, 84.92 ± 13.86 nM versus SLN TG, 74.24 ± 13.95 nM; n = 4; p < NS).

SLN TG Hearts Showed a Decreased Cardiac Performance in Isolated Work-performing Heart Preparations—The functional consequences of SLN overexpression in the heart were determined by measuring indices of cardiac performance with the anterograde-perfused work-performing heart preparations. The SLN TG hearts showed significant decreases in the maximum rate of contraction (+dP/dt) and relaxation (−dP/dt) compared with NTG hearts (Table 1). A tendency toward decreased baseline systolic and diastolic pressure was also observed in TG hearts; however, these decreases were not statistically different from NTG hearts. The other parameters of cardiac function such as time to peak pressure and half-relaxation pressure derived from intraventricular pressure tracings were not altered (Table 1).
in TG versus NTG mice. Force-frequency (FF) behavior in NTG mice was biphasic. At lower rates of stimulation, a small positive FF relationship was observed, after which force declined upon further increase in stimulation frequency. In SLN TG mice, the loss of developed force with increasing frequency was larger, resulting in lower force development compared with NTG mice at 12 and 14 Hz. From Fig. 6A, it can be seen that at 4 Hz, the speed of contraction was slower in TG mice at low frequency, but not different at 14 Hz.

To determine the effect of isoproterenol on muscle contraction, a typical isoproterenol dose-response behavior was recorded. Under maximal and near-maximal concentration, force development was impaired in TG muscle compared with NTG. However, at maximal isoproterenol stimulation, the differences noted for the speed of contraction and relaxation, observed at 4 Hz between the groups became insignificant (Fig. 6B).

In addition, RCC experiments were carried out to determine the SR Ca\(^{2+}\) load. At a baseline frequency of 4 Hz, TG mice showed an RCC amplitude of 7.29 ± 1.76 mN/mm\(^2\) compared with 11.65 ± 1.62 mN/mm\(^2\) in NTG mice (NS, \(p = 0.12\)). At a stimulation of 12 Hz, TG mice showed a decreased RCC amplitude of 6.13 ± 1.56 mN/mm\(^2\) (\(n = 6\)), compared with the NTG littermates (11.03 ± 1.19 mN/mm\(^2\), \(n = 7\), \(p < 0.05\)). These data suggest that overexpression of SLN decreases SR calcium load.

**Overexpression of SLN Decreases Ca\(^{2+}\) Transient Amplitude and Slows Relaxation in Ventricular Myocytes**—The effect of SLN overexpression on myocyte Ca\(^{2+}\) handling was studied using isolated ventricular myocytes from TG and NTG hearts (Fig. 7). Myocytes from SLN TG ventricles showed a decrease in Ca\(^{2+}\) transient amplitude by 57% (Fig. 7B) and significantly prolonged the rate of relaxation (NTG, 163 ± 14 ms versus TG, 255 ± 16 ms). In addition, shortening data paralleled the Ca\(^{2+}\) transient data (data not shown). Interestingly, \(\beta\)-adrenergic stimulation with isoproterenol restored calcium transient amplitude in TG myocytes to the same level as NTG control myocytes (Fig. 7, A and B). Myocyte relaxation was also similar after isoproterenol stimulation (Fig. 7C). We further investigated the force-frequency relationship in myocytes isolated from SLN TG and NTG ventricle. At lower frequencies, there was a significant difference in Ca\(^{2+}\) transient amplitude and relaxation (0.2 Hz, NTG: 429 ± 31 ms versus SLN TG, 520 ± 43 ms, \(p < 0.05\)). However, at higher frequencies, the relaxation times were similar in both groups (2 Hz, NTG: 170 ± 7 ms versus SLN TG, 171 ± 4 ms, \(p = \text{NS}\)). In addition, Ca\(^{2+}\) transient amplitude and shortening at lower frequencies was significantly reduced in TG versus NTG myocytes, but similar at higher frequencies (data not shown).

**DISCUSSION**

To better define the role of SLN in cardiac physiology, we chose to increase the ratio of SLN to SERCA2a in the ventricle (because it is naturally low) and study how an increase in SLN affects calcium homeostasis and heart function. The major finding of our study is that overexpression of SLN in the ventricle resulted in decreased SERCA pump affinity for calcium, Ca\(^{2+}\) transient amplitude and shortening, and slowed relaxation. More importantly, our data indicate that the inhibitory effect of SLN can be reversed by the \(\beta\)-adrenergic agonist, isoproterenol, suggesting that SLN acts as a reversible inhibitor similar to PLB.

Our studies additionally confirm many observations reported by Asahi et al. (12), in which rabbit SLN was expressed in the mouse by targeting a single copy of the \(\alpha\)-MHC-NF-SLN construct into the \(Hprt\) locus of the x chromosome. Targeting of the SLN into the \(Hprt\) locus resulted in heterogeneous SLN expression in female mice because of \(x\)-inactivation. This study showed that overexpression of rabbit SLN in

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**TABLE 1**

| Parameters | NTG, \(n = 6\) | SLN TG, \(n = 5\) |
|------------|----------------|-----------------|
| +dP/dt\(^{a}\), mmHg/s | 3835 ± 309 | 3115 ± 171\(^{a}\) |
| −dP/dt\(^{a}\), mmHg/s | 3738 ± 261 | 2911 ± 279\(^{b}\) |
| SP\(^{a}\), mmHg | 111.3 ± 6.5 | 106.0 ± 5.9 |
| DP\(^{b}\), mmHg | −12.1 ± 1.1 | −10.5 ± 1.23 |
| EDP\(^{b}\), mmHg | 8.1 ± 2.5 | 6.4 ± 2.8 |
| TPP\(^{b}\), ms/mmHg | 0.40 ± 0.03 | 0.43 ± 0.05 |
| TR1/2\(^{b}\), half relaxation pressure, normalized to one-half relaxation pressure | 0.53 ± 0.08 | 0.54 ± 0.11 |

\(^{a}\) Significantly different from NTG.

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**FIGURE 4.** Calcium uptake function in NTG and SLN TG mice. Ca\(^{2+}\) uptake assays were performed using whole heart homogenates from 24-week-old mice (\(n = 4\) for each group). The \(V_{\text{max}}\) of Ca\(^{2+}\) uptake was obtained at pCa 6.0 in both groups.
mouse hearts reduced the apparent Ca$^{2+}$ affinity of the SERCA pump. *In vivo* measurements of cardiac function showed significant decreases in $+\frac{dP}{dt}$ and $-\frac{dP}{dt}$ and leads to ventricular hypertrophy. They concluded that SLN inhibits the SERCA pump by stabilizing SERCA2a-PLB interaction and inhibiting PLB phosphorylation. This inhibition could be reversed upon $\beta$-adrenergic agonist-mediated PLB phosphorylation. These studies taken together allowed us to reach similar conclusions that SLN overexpression leads to decreased SERCA2a pump affinity and contractile function of the heart. In contrast, the overexpression of mouse SLN in the mouse ventricle described here did not induce cardiac hypertrophy/heart failure. The development of cardiac hypertrophy observed by Asahi *et al.* (12) may very well be caused by the overexpression of rabbit SLN in the mouse heart, which differs from the mouse, at the N-terminal region (13).

The finding that overexpression of SLN results in decreased pump affinity for Ca$^{2+}$ and depressed SR Ca$^{2+}$ load (as measured by RCC experiments) suggests that SLN is an inhibitor of the SERCA pump. This is further supported by decreased Ca$^{2+}$ transient amplitudes and slowed rates of relaxation in isolated myocytes. Consistent with the myocyte studies, both $+\frac{dP}{dt}$ and $-\frac{dP}{dt}$ were found to be significantly decreased in the SLN TG heart as assessed by the hemodynamic measurements. Similar observations have also been made in muscle.
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![Graphs showing Ca^2+ transients in myocytes isolated from NTG and SLN TG hearts.](image)

Preparations. These results corroborate nicely with our recent report on myocyte contractility and Ca^{2+} transients using adenoviral-mediated SLN overexpression in rat ventricular myocytes (11). These functional changes, however, are not sufficient to cause any cardiac pathology. It is quite likely that some of these functional changes could be compensated by mechanisms yet to be determined.

An interesting finding of this study is that the inhibitory effect of SLN on contractile function could be reversed by treatment with isoproterenol and at high frequency. MacLennan and co-workers (12) also reported similar findings in SLN TG hearts, where isoproterenol restored contractile function. They reported that basal PLB phosphorylation was decreased in SLN TG hearts and upon adrenergic stimulation, PLB phosphorylation was restored to the same extent as control hearts. The authors interpreted that enhanced PLB phosphorylation (which may result in dissociation of SLN from PLB) could be the mechanism for restoration of function in SLN TG hearts during β-adrenergic stimulation. However, in the present study, we did not see an appreciable difference in the β-adrenergic agonist-mediated phosphorylation of PLB between the NTG and TG ventricle. These results suggest that SLN could play a direct role in mediating the β-adrenergic response in the SLN TG hearts. SLN has a conserved threonine (Thr5) residue at the N terminus that can be phosphorylated during β-adrenergic stimulation by serine/threonine kinases such as CaMKII, which may relieve its inhibitory effect on SERCA pump. Mutation of Thr5 to Ala leads to a slight gain in inhibitory function (18) further supporting the idea that phosphorylation of Thr5 could play a role in regulating SLN function. Recent studies also suggest that there are additional mechanisms independent of PLB phosphorylation, which may play a significant role in mediating the positive inotropic effects of the β-adrenergic agonist (30) and force-frequency-dependent relaxation (31). However, the potential role of Thr5 as a target for calcium/calmodulin-dependent protein kinase II or protein kinase A phosphorylation during β-adrenergic stimulation and increasing frequency needs to be demonstrated.

MacLennan and co-workers (9, 20) have demonstrated that SLN can form a binary complex with PLB. In a recent study we have also observed that PLB can be co-immunoprecipitated with NF-SLN (11). These studies suggest a physical interaction between SLN and PLB, and such a binary complex may enhance its inhibitory effect on SERCA (9). Phosphorylation of PLB during β-adrenergic stimulation could dissociate the PLB-SLN binary complex from SERCA2a as effectively as it would remove PLB alone. It has also been suggested that SLN interaction with PLB could prevent PLB polymerization resulting in an increase in the active form, the monomer (8) thus promoting super-inhibition of SERCA pump. However, this model does not apply to situations where PLB is very low (atria) or non-existent such as in fast-twitch skeletal muscle. Further, our published data (11) and results from this study show that overexpression of SLN did not alter PLB levels, monomer to pentamer ratio, or its phosphorylation status. Therefore it is unlikely that the SLN effect is mediated by monomerization of PLB. However, this does not exclude the possibility that SLN could influence the inhibitory action of PLB, by binding allosterically to the same or to a different site. It should also be taken into account that SLN is expressed at higher levels in fast-skeletal muscles of larger mammals, where PLB is absent, which readily suggests that SLN can regulate SERCA pump, independent of PLB. Future studies will be directed toward understanding the mechanism of SLN action on the SERCA pump in the absence of PLB.

In conclusion, we found that overexpression of SLN in mouse hearts decreases both SR calcium handling and cardiac contractility. The inhibitory effect of SLN can be relieved upon β-adrenergic receptor stimulation and increased frequency, suggesting that it is a reversible inhibitor. The decreased contractility observed in SLN TG hearts is primarily caused by changes in the SLN/SERCA ratio, not alteration in PLB expression or phosphorylation status. Taken together our results suggest that SLN is a novel regulator of SERCA pump and plays an important role in cardiac physiology.

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