Enhanced resistance to fatigue and altered calcium handling properties of sarcalumenin knockout mice

Xiaoli Zhao, Morikatsu Yoshida, Leticia Brotto, Hiroshi Takeshima, Noah Weisleder, Yutaka Hirata, Thomas M. Nosek, Jianjie Ma, and Marco Brotto

1Department of Physiology and Biophysics, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey; 2Department of Medical Chemistry, Tohoku University School of Medicine, Sendai, Miyagi, Japan; and 3Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio

Submitted 24 January 2005; accepted in final form 27 June 2005

Zhao, Xiaoli, Morikatsu Yoshida, Leticia Brotto, Hiroshi Takeshima, Noah Weisleder, Yutaka Hirata, Thomas M. Nosek, Jianjie Ma, and Marco Brotto. Enhanced resistance to fatigue and altered calcium handling properties of sarcalumenin knockout mice. Physiol Genomics 23: 72–78, 2005. First published July 5, 2005; 10.1152/physiolgenomics.00020.2005.—Sarcalumenin is a Ca<sup>2+</sup>-binding protein located in the sarcoplasmic reticulum of striated muscle cells, the physiological function of which has not been fully determined yet. Using sarcalumenin knockout (sar+/−) mice, we showed that sar ablation altered store-operated Ca<sup>2+</sup> entry (SOCE) and enhanced muscle fatigue resistance. Sar+/− mice fatigued less with treadmill exercise, and intact isolated soleus and extensor digitorum longus muscles from sar+/− mice were more resistant to intermittent fatiguing stimulation than those from wild-type mice. Enhanced SOCE was observed in the sar+/− muscles. Biochemical analysis revealed that sar+/− muscles contained significantly elevated expression of mitsugumin 29 (MG29), a synaptophysin-related membrane protein located in the triad junction of skeletal muscle. Because the ablation of mg29 has been shown to cause increased fatigability and dysfunction of SOCE, the enhanced SOCE activity seen in sar+/− muscle may be correlated with the increased expression of MG29. Our data suggest that systemic ablation of sarcalumenin caused enhanced resistance to muscle fatigue by compensatory changes in Ca<sup>2+</sup> regulatory proteins that effect SOCE.

muscle fatigue; mitsugumin 29; store-operated calcium channel; excitation-contraction coupling; skeletal muscle

MUSCLE FATIGUE IS DEFINED as reduced muscle force production or capacity to do work after prolonged activity from exercise or repetitive electrical stimulation of isolated muscle preparations. The major factors influencing muscle fatigue include the integrity of triad junctional structures, the amount of Ca<sup>2+</sup> released from the sarcoplasmic reticulum (SR) into cytosol during each contraction and contractile protein modifications (1). We have previously demonstrated that store-operated Ca<sup>2+</sup> entry (SOCE) also played an important role in muscle fatigue and aging (3). The cumulative entry of Ca<sup>2+</sup> through the store-operated Ca<sup>2+</sup> channel (SOC) not only provides the ideal mechanism for refilling of intracellular Ca<sup>2+</sup> stores in skeletal muscle cells (7), but it also can certainly add to Ca<sup>2+</sup> needed for muscle contraction under conditions of fatigue during intensive exercise when the intracellular stores of Ca<sup>2+</sup> become depleted.

Sarcalumenin (SAR) is a low-affinity, high-capacity, Ca<sup>2+</sup>-binding protein (CBP) within the longitudinal SR of skeletal and cardiac muscles (12). The apparent molecular mass of SAR is 160 kDa, and its NH<sub>2</sub> terminus contains an acidic Ca<sup>2+</sup>-binding region similar to that of calsequestrin (CsQ) (11), the major CBP residing in terminal cisternae of skeletal muscle SR. Previous studies indicate that SAR may have multiple and context-dependent functions. First, SAR colocalized with SR endoplasmic reticulum (ER) Ca<sup>2+</sup>-ATPase (SERCA) at the longitudinal SR (12) and accelerated Ca<sup>2+</sup> transport (23), which implied the role of SAR in Ca<sup>2+</sup> shuttling. Second, in smooth muscle cells, SAR expression levels gradually increased during muscle maturation, suggesting a function in development (19). Third, SAR levels and those of the other triad proteins, such as ryanodine receptor (RyR), dihydropyridine receptor (DHPR), SERCA1, and triadin, decreased sharply after chronic low-frequency stimulation in extensor digitorum longus (EDL) muscle, whereas the protein levels of SERCA2 and calreticulin increased (16). This is significant, because it implies that muscles that are generally more fatigue resistant (a well known adaptation to take place in fast-twitch muscles after chronic low-frequency stimulation) display lower SAR expression levels as part of the compensatory mechanisms involved with fiber type switching. Fourth, interestingly, the expression level of SAR in the muscle fibers of X chromosome-linked, dystrophin-deficient muscular dystrophy (mdx) mouse model was reduced by 70% (5). While this data may suggest that SAR functions to maintain muscle Ca<sup>2+</sup> homeostasis and normal contraction in dystrophic muscles, the response in this animal model is significantly complicated by the generalized muscle wasting process, and downregulation of SAR in this animal model could also be the result of the exacerbated muscle injury that occurs in these muscles. Fifth, it is also important to note that SR CBPs do not simply increase the luminal Ca<sup>2+</sup> storage capacity by ion trapping or facilitate the Ca<sup>2+</sup> uptake by lowering the concentration of free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) in SR; they act as endogenous regulators of SOCE via retrograde signaling pathways (20). Most recently, Yoshida et al. (23) generated a SAR-deficient mouse strain and found that the mutant skeletal muscle had fenestrated SR ultrastructures while retaining normal force generation. A marginally weakened Ca<sup>2+</sup> uptake activity was detected in the SR prepared from mutant muscle, indicating that SAR contributes to Ca<sup>2+</sup> buffering in the SR lumen and also to the maintenance of Ca<sup>2+</sup> pump proteins.

Our study explored the effects of systemic sar ablation on muscle performance and assessed its function in regulating excitation-contraction coupling (E-C coupling). We tested the
fatigability of control and systemic sar knockout mice (sar\(^{+/−}\))
and found that the ablation of this protein significantly enhanced muscle performance both in vivo (i.e., treadmill exercise) and during in vitro low-frequency, intermittent fatiguing stimulation. Furthermore, this fatigue-resistant phenotype of isolated muscles from sar\(^{+/−}\) mice was dependent on the presence of extracellular [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{o}\)). This dependence was linked to enhanced SOCE function and increased expression of mitsugumin 29 (MG29) in sar\(^{+/−}\) muscle.

**MATERIALS AND METHODS**

*Generation of SAR-deficient mice.* The transgenic mice were generated as described before (23). Briefly, the targeting vector was constructed by cloning of sar genomic DNA fragments into pBlue-script SK\(^−\) vector (Stratagene). A 1.1-kb neomycin-resistant gene (neo) was inserted into exon 1 of the sar gene, and a 0.7-kb diphtheria toxin gene was attached at the end of the genomic fragment. ES cells were transfected with the targeting vector and cultured in the presence of G418. The first exon of sar was replaced by neo through homologous recombination with targeting vector at the sar locus. Chimaeric male mice were generated by injecting the ES cells into C57BL/6J blastocysts, and bred to yield mice heterozygous for the sar allele. Homologous sar knockout mice were obtained by crossing the heterozygous mice. The SAR-deficient mice used in this study were backcrossed to C57BL/6J mice for at least six generations.

*Treadmill performance.* Three-month-old male sar\(^{+/−}\) mice and wild-type C57BL/6J mice (body wt, 34 g) were subjected to a treadmill running test, conducted following a protocol modified from that of Massett and Berk (13). Mice were placed on a rodent treadmill equipped with an electric grid at the rear and were allowed to acclimatize for four consecutive days. On day 1, they ran at a speed of 38 m/min for 5 min; on day 2, 48 m/min for 5 min; on day 3, 58 m/min for 5 min; and on day 4, 68 m/min for 5 min. On day 5, control and sar\(^{+/−}\) mice ran concomitantly at 88 m/min until exhaustion (indicated by falling on the electric grid twice), and running times until exhaustion were recorded. Two separate trials with three wild-type and three sar\(^{+/−}\) mice per trial were conducted. All six sar\(^{+/−}\) mice ran significantly longer than their wild-type controls.

*Intact muscle preparation.* As previously described (15), wild-type and mutant EDL and soleus (SOL) muscles were dissected intact and placed into 2.5 mM Ca\(^{2+}\) modified Ringer solution (142 mM NaCl, 5 mM KCl, 2.5 mM CaCl\(_2\), 2 mM MgCl\(_2\), 10 mM HEPES, 10 mM glucose, pH 7.4) bubbled with 100% O\(_2\). We also compared our results using a bicarbonate buffer system and did not observe significant differences. In addition, we used a HEPES buffered system to more closely match the conditions of experiments performed in dissociated and skinned muscle cells.

After careful dissection of the intact muscles, a pair of control and mutant intact muscles was mounted vertically onto 20-ml Radnoti glass chambers with built-in platinum stimulating electrodes (Monrovia, CA). One tendon of the muscle was attached to a force transducer and the other one to a stationary arm in preparation for in vitro fatiguing experiments. Muscles used in a Western blotting assay were placed in 100 \(\mu\)l of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 2% CHAPS) with 1% protease inhibitor cocktail (PIC, Sigma), flash frozen by liquid nitrogen for 5 min, and stored at −80°C until use.

*In vitro fatiguing stimulation protocol.* The fatiguing protocol used in this study was modified from our previous protocol (4, 14, 15). A Powerlab computer-interface program (ADInstruments) was used to control the electrical stimulation protocols and to record, digitize, and store force output data. Field stimulation (squared-waves electrical currents of 500-ms duration, 300 mA using frequencies of stimulation in the range of 1−120 Hz) was accomplished with platinum electrodes running on both sides of intact muscles. After mounting, wild-type and sar\(^{+/−}\) muscles were mounted in parallel, and their resting lengths were adjusted to produce maximal isometric force (T\(_{\text{max}}\)). The muscles were then subjected to the force vs. frequency relationship, and the stimulating frequencies that produced T\(_{\text{max}}\) (high frequency) and 50% T\(_{\text{max}}\) (low frequency) were determined. Frequencies that produced T\(_{\text{max}}\) and 50% T\(_{\text{max}}\) were then employed for the remaining of the protocols. After 20 min of equilibration (1-min interval, 0.83% duty cycle) at high frequency, the bathing solution was changed to 2.5 mM Ca\(^{2+}\) Ringer or 0 mM Ca\(^{2+}\) Ringer (142 mM NaCl, 5 mM KCl, 0.2 mM EGTA, 2 mM MgCl\(_2\), 10 mM HEPES, 10 mM glucose, pH 7.4), and muscles were exposed to a fatiguing stimulation protocol (1-s interval, 50% duty cycle) using the low-frequency (i.e., 50% T\(_{\text{max}}\)) stimulation for 5 min. Force output data during fatigue were normalized to 50% T\(_{\text{max}}\) before the onset of fatigue and analyzed by Origin software (OriginLab). All experiments were conducted at room temperature (23 ± 2°C).

*Skinned muscle fiber preparation and SOCE measurement.* We developed a novel method for the direct visualization of SOCE in cultured adult mammalian EDL muscle fibers that was inspired by the elegant studies of Lamont and coworkers (10). Single intact muscle fibers were dissociated from the EDL of control and sar\(^{+/−}\) mice. The tendons were kept intact on both ends, and fibers were kept under culturing in DMEM with 2% horse serum and 1% penicillin-streptomycin (Gibco BRL) for 72 h in an incubator at 5% CO\(_2\) and 37°C. Immediately before confocal measurement, a single muscle fiber was bathed in skinnning solution (500 \(\mu\)M Rhod-5N, 400 \(\mu\)M CaCl\(_2\), 140 mM K-glutamate, 6.5 mM MgCl\(_2\), 3 mM creatine phosphate, 5 mM ATP, 20 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), pH 7.1) and mechanically skinned with microforceps, allowing transverse tubules (T-tubules) to automatically re-seal and to trap the Rhod-5N conjugated inside the T-tubules. Skinned fibers were dissociated by 10.2 ± 0.3 on June 19, 2017 http://physiolgenomics.physiology.org/ Downloaded from Physiol Genomics • VOL 23 • www.physiolgenomics.org  by 10.220.33.2 on June 19, 2017.
Twenty micrograms of protein were loaded on 8–12% NuPage Bis-Tris gel (Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membrane. Proteins were detected by anti-mouse monoclonal antibodies as follows: triadin, junctophilin-1 (JP1) (21), α1-subunit of DHPR, CsQ1, α-actin, SERCA2, SAR (antibody detects both 160-kDa SAR and 53-SRGP/53-kDa SR glycoprotein, the alternative splicing product of SAR mRNA), MG29, RyR1, Grp78 (glucose-regulated protein/BiP, ER/SR stress protein), and GAPDH. HITACHI GeneTools software was used to quantify protein band intensities. For statistical analysis of protein levels, densitometry levels were determined on HITACHI SynGene system with GeneTools software and were normalized to GAPDH levels. The mean values of wild-type and knockout mice were compared, with wild-type protein expression levels set to be 1.

Statistics. Data shown are expressed as means ± SE. Because data were normally distributed, Student’s t-tests were applied for comparison between sar/−/− and wild-type groups. No additional statistical analyses were used for the force vs. frequency curves, because such relationships were essentially identical among experimental groups.

RESULTS

Sar/−/− mice fatigue resistance depends on extracellular Ca2+. During the treadmill performance studies, sar/−/− mice displayed increased running time to exhaustion (65.3 ± 8.7 min) compared with wild-type animals (32.3 ± 8.9 min) (Fig. 1). For in vitro intact muscle fatigue studies, in 2.5 mM Ca2+ Ringer bathing solution, both EDL (Fig. 2A) and SOL (Fig. 2B) from sar/−/− mice were more resistant to low-frequency intermittent fatiguing stimulation, which was consistent with our in vivo findings.

Fig. 2. Systemic ablation of sar enhanced muscle resistance to low-frequency intermittent fatiguing stimulation in 2.5 mM extracellular Ca2+ concentration ([Ca2+]o). A and B: contractile forces from intact, isolated muscles of WT (gray) and sar/−/− mice (black) were recorded upon fatiguing stimulation with Ca2+ present in extracellular solution. Forces were normalized to the control force immediately before the onset of fatigue stimulation [50% maximal isometric force (Tmax)]. A: contractile forces (50% Tmax) from extensor digitorum longus (EDL), stimulated at 40 Hz/s for 5 min. B: contractile forces (50% Tmax) from soleus muscle (SOL), stimulated at 25 Hz/s for 5 min. C: sustained force (Yo) of sar/−/− (solid bar) and WT SOLs (open bar) in 2.5 mM [Ca2+]o. * *P < 0.01 vs. WT. D: fast component of force decay (A1) of sar/−/− and WT SOLs in 2.5 mM [Ca2+]o. **P < 0.01 vs. WT. Both A and B were normalized to 50% Tmax just before fatigue onset; n = 13.
To quantify the time-dependent changes in force output, a two-exponential decay function was used to fit the curve: \[ Y(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + Y_0, \]
where \( A_1 \) represents the amplitude of the fast-fatiguing component with half-time constant \( \tau_1 \), \( A_2 \) represents the slow-fatiguing component with half-time constant \( \tau_2 \), and \( Y_0 \) illustrates the sustained fraction of force output at the end of the 5-min fatigue stimulation. Clearly, \( Y_0 \) for the mutant muscles was significantly larger than that for the wild-type control muscles, suggesting that the mutant muscles were more resistant to fatigue (Fig. 2C).

Moreover, the fast-fatiguing component was significantly less in the mutant muscle compared with the wild-type control muscles (Fig. 2D).

To investigate whether the change of fatigability in mutant mice is related to Ca\(^{2+}\) handling alterations, extracellular Ca\(^{2+}\) in the bathing solution was removed, while fatiguing isolated EDL and SOL muscles. The improvement of muscle performance from sar ablation over wild-type mice was totally abolished (Fig. 3). Mutant and wild-type contractility profiles overlapped with each other after removal of Ca\(^{2+}\). The exponential fit of \( Y_0, A_1 \) (Fig. 3, C and D), \( A_2, \) and \( \tau_1 \) and \( \tau_2 \) (data not shown) of sar\(^{-/-}\) muscles revealed no statistic difference from wild-type muscles in 0 mM Ca\(^{2+}\) Ringer. These results suggested that the fatigue-resistance phenotype generated by systemic sar ablation was totally dependent on entry of Ca\(^{2+}\) from the extracellular milieu. The force vs. frequency relationships in either 2 or 0 mM extracellular Ca\(^{2+}\) were not signif-

Fig. 3. Similar muscle performance from WT and sar\(^{-/-}\) muscle at 0 mM [Ca\(^{2+}\)]\(_o\) condition. A: contractile forces (50% \( T_{\text{max}} \)) recorded from a pair of WT (gray) and sar\(^{-/-}\) (black) EDL muscles in 0 mM [Ca\(^{2+}\)]\(_o\), subjected to identical stimulation protocol as in Fig 2. B: contractile forces (50% \( T_{\text{max}} \)) from SOL muscles in 0 mM [Ca\(^{2+}\)]\(_o\). C: sustained force (\( Y_0 \)) of sar\(^{-/-}\) (solid bar) and WT SOLs (open bar) in 0 mM [Ca\(^{2+}\)]\(_o\). D: fast component of force decay (\( A_1 \)) of sar\(^{-/-}\) and WT in 0 mM [Ca\(^{2+}\)]\(_o\); n = 6.

Fig. 4. Similar force vs. frequency relationship for sar\(^{-/-}\) and WT. WT and sar\(^{-/-}\) muscles were mounted in parallel, and their resting lengths were adjusted to produce \( T_{\text{max}} \). Field stimulation (squared-waves electrical currents of 500 ms duration and 300 mA) was accomplished with platinum electrodes running on both sides of intact muscles. The muscles were then subjected to the force vs. frequency relationship using frequencies of stimulation in the range of 1–120 Hz. Traces are as follows: WT muscles in 2 mM Ca\(^{2+}\) (●), sar\(^{-/-}\) muscles in 2 mM Ca\(^{2+}\) (○), WT muscles in 0 mM Ca\(^{2+}\) (□), and sar\(^{-/-}\) muscles in 0 mM Ca\(^{2+}\) (△).
icantly different between sar<sup><mo>−/−</mo></sup> and wild-type muscles (Fig. 4), and no differences for the T<sub>max</sub> of sar<sup><mo>−/−</mo></sup> and wild-type muscles were observed (data not shown).

Enhanced SOCE in sar<sup><mo>−/−</mo></sup> mice. Because the increased fatigue resistance was abolished on removal of Ca<sup>2+</sup> from the extracellular milieu, and because SOCE is responsible for refilling the SR Ca<sup>2+</sup> store, we hypothesized that the Ca<sup>2+</sup>-dependent fatigue resistance of sar<sup><mo>−/−</mo></sup> mice might involve modification of SOCE. SOCE function was evaluated by the skinned single muscle fiber technique, a unique, direct model for SOCE assessment. Confocal images clearly demonstrated that Rhod-5N fluorescence decreased only after depletion of SR Ca<sup>2+</sup> store with the application of TG and caffeine (Fig. 5). Rhod-5N was trapped in resealed T-tubule, giving the muscle fiber a compartmentalized appearance (Fig. 5A). Figure 5B shows the Rhod-5N intensity traces of wild-type and sar<sup><mo>−/−</mo></sup> deskinned fibers, normalized to the individual maximal loading (i.e., after T-tubule and SR loading). At point a (time = 0 s), the normalized Rhod-5N intensity of sar<sup><mo>−/−</mo></sup> and wild-type fibers was 0.80 ± 0.01 and 0.85 ± 0.02, respectively, while at point b (time = 262 s), those values were 0.97 ± 0.02 and 0.98 ± 0.01. There were no significant differences between sar<sup><mo>−/−</mo></sup> and wild-type fibers at these data points, suggesting that Rhod-5N had identical spectral properties in both the wild-type and mutant muscles. However, SOCE activity was elevated in sar<sup><mo>−/−</mo></sup> fibers, as the Rhod-5N intensity of sar<sup><mo>−/−</mo></sup> muscle dropped to 0.45 ± 0.02 and the corresponding wild-type value only dropped to 0.55 ± 0.03 (P < 0.05). The smaller value for sar<sup><mo>−/−</mo></sup> fibers indicated that more Ca<sup>2+</sup> had been transported out of the T-tubes via enhanced SOC activity after depletion of the SR Ca<sup>2+</sup> stores. The faster and greater decrease of Rhod-5N intensity from the T-tubules of sar<sup><mo>−/−</mo></sup> fibers after depletion of SR Ca<sup>2+</sup> store provided direct evidence that SOCE was enhanced in muscles from the mutant mice.

Compensatory elevation of MG29 in sar<sup><mo>−/−</mo></sup> muscles. Our functional studies did not allow us to determine whether the gain of SOCE function in sar<sup><mo>−/−</mo></sup> muscle is a direct effect of the loss of SAR protein. Alteration of other triad components may contribute to this elevated SOCE. We tested the expression levels of a series of E-C coupling relevant triad junction proteins and SR resident proteins (Fig. 6, A and B). We found that, on average, the MG29 expression level of sar<sup><mo>−/−</mo></sup> muscles increased to 1.7 ± 0.2 compared with that of the wild-type muscles. SERCA2 level was marginally decreased in mutant muscles, but it was not statistically different from that of the wild type (P > 0.05). No significant changes of other Ca<sup>2+</sup> channel (SOC) function were observed.

Fig. 5. Enhanced store-operated Ca<sup>2+</sup> entry (SOCE) function was observed in sar<sup><mo>−/−</mo></sup> muscle fibers. A: confocal images of WT and sar<sup><mo>−/−</mo></sup> EDL. Initial Rhod-5N-Ca<sup>2+</sup> loading at time (t) = 0 s (a), after maximal transverse tubule (T-tubule) and sarcoplasmic reticulum (SR) loading at t = 262 s (b), and after depletion of SR Ca<sup>2+</sup> store by thapsigargin (TG)-caffeine at t = 1,000 s (c). B: measurement of WT (green) and sar<sup><mo>−/−</mo></sup> (red) EDL, store-operated Ca<sup>2+</sup> channel (SOC) function as reflected by the decrease in Rhod-5N fluorescence intensity. Data were normalized to maximal intensity at point b (mean ± SE), n = 6 for both WT and sar<sup><mo>−/−</mo></sup>, which was essentially identical for both muscles. From each muscle fiber, the mean value of 6 T-tubules was obtained and a global mean value from the 6 experiments was calculated and statistically compared.

Fig. 6. Compensatory elevation of mitsugumin 29 (MG29) in sar<sup><mo>−/−</mo></sup> muscles. A: Western blotting assay of Ca<sup>2+</sup> regulatory proteins in skeletal muscles from WT and sar<sup><mo>−/−</mo></sup> mice. B: relative change of protein expression levels in sar<sup><mo>−/−</mo></sup> muscles compared with WT control was averaged from 13 pairs of muscle preparations. Densitometry scans of Western blot were first normalized to the GAPDH levels to ensure equal loading of proteins. The relative protein expression value from sar<sup><mo>−/−</mo></sup> muscles (hatched bars) were then divided by that value from WT (open bars) controls. Data were presented as means ± SE. Other than the significant elevation of MG29 (*P < 0.05) in sar<sup><mo>−/−</mo></sup> muscles, other protein expression levels did not change. SAR, sarcalumenin; DHPR, dihydropyridine receptor; RyR, ryanodine receptor; JP<sub>1</sub>, junctophilin-1; SERCA, SR/ER Ca<sup>2+</sup>-ATPase; CsQ, calsequestrin.
SAR protein was originally identified 15 years ago as a CBP located at the inner membrane of longitudinal SR in skeletal muscle cells (11). However, rarely has investigation of SAR function occurred, possibly because most Ca\(^{2+}\) ions inside SR were bound to terminal cisternae by CsQ; thus tracks of Ca\(^{2+}\) were rarely seen around longitudinal SR (6). In addition, while the NH\(_2\) terminus of SAR has negative amino acid clusters as a putative Ca\(^{2+}\)-binding site, this region is poorly conserved among species (12). These observations limited further steps to determine the function of SAR. In this study, we investigated the fatigability of systemic sar-/- muscles in a mouse model (23). For in vitro fatiguing experiments, a low-frequency intermittent stimulation protocol was used to mimic the physiological effects of intermittent fatigue. Also, we have previously demonstrated that this protocol preferentially evaluates SR Ca\(^{2+}\) store function over the properties of the contractile machinery (3). We found that sar ablation produces a fatigue-resistant phenotype that depends on [Ca\(^{2+}\)]\(_o\). The dependence of sar-/- fatigue resistance on [Ca\(^{2+}\)]\(_o\) suggested that the SOCE might play a role in this phenomenon. Inspired by the elegant works of Launikonis and coworkers (8–10), we directly monitored, for the first time, SOCE in skinned muscle fibers from adult cultured EDL muscles from both wild-type and sar knockout mice. Indeed, our further investigation revealed that sar-/- muscle had enhanced SOCE function and an elevated MG29 expression level, possibly as a long-term compensatory response to sar ablation.

Our hypothesis is as shown in Fig. 7, that the enhanced SOCE is a result of chronic adaptation from sar ablation, with the involvement of enhanced MG29 level. SAR was reported to be located at longitudinal SR and interacts with SERCA, the Ca\(^{2+}\) pump responsible for moving Ca\(^{2+}\) from cytosol back to SR. The capacity of SAR to bind Ca\(^{2+}\) is 35 M Ca\(^{2+}\)/M protein (12), whereas that of CsQ is ~50; this property is consistent with that of a Ca\(^{2+}\) transporter rather than Ca\(^{2+}\) storage. A recent study by Yoshida et al. (23) showed that sar ablation caused a weakened Ca\(^{2+}\) uptake in isolated SR vesicles. With loss of SAR, the SR Ca\(^{2+}\) store could be emptied to a greater extent or for a longer duration, and this signal in turn either facilitates the activation of SOCE or promotes a larger SOCE response (Fig. 7). An elevated expression of MG29 in sar-/- mice may also contribute to the enhanced function of SOCE. MG29 shares 45% homology with synaptophysin, a protein known to be responsible for the formation of synapses in neuron (22). Our previous studies showed that MG29 null muscles were more susceptible to fatigue (14), a phenotype caused by alteration within the E-C coupling machinery (2). MG29 can also interact with RyR and activate SR Ca\(^{2+}\) release (17). Thus the increased expression level of MG29 in sar-/- muscles might contribute to enhanced Ca\(^{2+}\) release from the SR or a more efficient E-C coupling, which also leads to a more depleted SR store and hence fully activated SOCE. In addition, muscle cells lacking mg29 gene displayed impaired SOCE and intracellular Ca\(^{2+}\) homeostasis defects (18), implying MG29 might have a direct role on SOCE (Fig. 7).

The fatigue-resistant phenotype could be an integrative result from multiple alterations in sar-/- muscle. First, because SOCE is responsible for refilling Ca\(^{2+}\) from the extracellular compartment into the cytosol after depletion of SR Ca\(^{2+}\) store in skeletal muscle cell (7), this ensures that there is enough Ca\(^{2+}\) available for each contraction cycle. During fatigue, even a small difference in Ca\(^{2+}\) entry via SOC will significantly influence muscle function, because, with every contraction cycle, if more Ca\(^{2+}\) enters the muscle cell, more Ca\(^{2+}\) will be available for the next contraction. Second, MG29 expression would result in an increased cytosolic Ca\(^{2+}\) level due to more efficient SR Ca\(^{2+}\) release and increased SR Ca\(^{2+}\) reservoir due to enhanced SOCE, enabling sar-/- muscles to have a more effective E-C coupling.

Systemic ablation of sar reduced the fatigability of skeletal muscles. Elevated SOCE activity in sar-/- muscle might account for this phenomenon. In addition, the enhanced SOC activity and increased resistance to fatigue were well correlated with a significant increase in the level of MG29, a protein thought to increase E-C coupling efficiency. Our studies may provide novel insights into impaired muscle performance under pathophysiological conditions. For example, the significant downregulation of SAR reported in muscular dystrophy mdx muscles (5) suggests that this protein could serve important compensatory roles in muscle disease. Our study also pioneered a novel method for detection of SOCE in mammalian muscle fibers and a unique transgenic model of enhanced muscle performance. In fact, the SAR-deficient mouse is the only model of enhanced fatigue resistance for which the protein of interest is an SR-resident protein. It will be necessary to investigate the effects of acute MG29 and sar ablation to determine their specific contributions to muscle function and to develop possible therapies for muscular dystrophy and for the prevention of fatigue-related decrease in muscle function. Our novel methodology in cultured mammalian muscle fibers should also allow for the differentiation between the

---

**Discussion**

SAR protein was originally identified 15 years ago as a CBP located at the inner membrane of longitudinal SR in skeletal muscle cells (11). However, rarely has investigation of SAR function occurred, possibly because most Ca\(^{2+}\) ions inside SR were bound to terminal cisternae by CsQ; thus tracks of Ca\(^{2+}\) were rarely seen around longitudinal SR (6). In addition, while the NH\(_2\) terminus of SAR has negative amino acid clusters as a putative Ca\(^{2+}\)-binding site, this region is poorly conserved among species (12). These observations limited further steps to determine the function of SAR. In this study, we investigated the fatigability of systemic sar-/- muscles in a mouse model (23). For in vitro fatiguing experiments, a low-frequency intermittent stimulation protocol was used to mimic the physiological effects of intermittent fatigue. Also, we have previously demonstrated that this protocol preferentially evaluates SR Ca\(^{2+}\) store function over the properties of the contractile machinery (3). We found that sar ablation produces a fatigue-resistant phenotype that depends on [Ca\(^{2+}\)]\(_o\). The dependence of sar-/- fatigue resistance on [Ca\(^{2+}\)]\(_o\) suggested that the SOCE might play a role in this phenomenon. Inspired by the elegant works of Launikonis and coworkers (8–10), we directly monitored, for the first time, SOCE in skinned muscle fibers from adult cultured EDL muscles from both wild-type and sar knockout mice. Indeed, our further investigation revealed that sar-/- muscle had enhanced SOCE function and an elevated MG29 expression level, possibly as a long-term compensatory response to sar ablation.

Our hypothesis is as shown in Fig. 7, that the enhanced SOCE is a result of chronic adaptation from sar ablation, with the involvement of enhanced MG29 level. SAR was reported to be located at longitudinal SR and interacts with SERCA, the Ca\(^{2+}\) pump responsible for moving Ca\(^{2+}\) from cytosol back to SR. The capacity of SAR to bind Ca\(^{2+}\) is 35 M Ca\(^{2+}\)/M protein (12), whereas that of CsQ is ~50; this property is consistent with that of a Ca\(^{2+}\) transporter rather than Ca\(^{2+}\) storage. A recent study by Yoshida et al. (23) showed that sar ablation caused a weakened Ca\(^{2+}\) uptake in isolated SR vesicles. With loss of SAR, the SR Ca\(^{2+}\) store could be emptied to a greater extent or for a longer duration, and this signal in turn either facilitates the activation of SOCE or promotes a larger SOCE response (Fig. 7). An elevated expression of MG29 in sar-/- mice may also contribute to the enhanced function of SOCE. MG29 shares 45% homology with synaptophysin, a protein known to be responsible for the formation of synapses in neuron (22). Our previous studies showed that MG29 null muscles were more susceptible to fatigue (14), a phenotype caused by alteration within the E-C coupling machinery (2). MG29 can also interact with RyR and activate SR Ca\(^{2+}\) release (17). Thus the increased expression level of MG29 in sar-/- muscles might contribute to enhanced Ca\(^{2+}\) release from the SR or a more efficient E-C coupling, which also leads to a more depleted SR store and hence fully activated SOCE. In addition, muscle cells lacking mg29 gene displayed impaired SOCE and intracellular Ca\(^{2+}\) homeostasis defects (18), implying MG29 might have a direct role on SOCE (Fig. 7).

The fatigue-resistant phenotype could be an integrative result from multiple alterations in sar-/- muscle. First, because SOCE is responsible for refilling Ca\(^{2+}\) from the extracellular compartment into the cytosol after depletion of SR Ca\(^{2+}\) store in skeletal muscle cell (7), this ensures that there is enough Ca\(^{2+}\) available for each contraction cycle. During fatigue, even a small difference in Ca\(^{2+}\) entry via SOC will significantly influence muscle function, because, with every contraction cycle, if more Ca\(^{2+}\) enters the muscle cell, more Ca\(^{2+}\) will be available for the next contraction. Second, MG29 expression would result in an increased cytosolic Ca\(^{2+}\) level due to more efficient SR Ca\(^{2+}\) release and increased SR Ca\(^{2+}\) reservoir due to enhanced SOCE, enabling sar-/- muscles to have a more effective E-C coupling.

Systemic ablation of sar reduced the fatigability of skeletal muscles. Elevated SOCE activity in sar-/- muscle might account for this phenomenon. In addition, the enhanced SOC activity and increased resistance to fatigue were well correlated with a significant increase in the level of MG29, a protein thought to increase E-C coupling efficiency. Our findings may provide novel insights into impaired muscle performance under pathophysiological conditions. For example, the significant downregulation of SAR reported in muscular dystrophy mdx muscles (5) suggests that this protein could serve important compensatory roles in muscle disease. Our study also pioneered a novel method for detection of SOCE in mammalian muscle fibers and a unique transgenic model of enhanced muscle performance. In fact, the SAR-deficient mouse is the only model of enhanced fatigue resistance for which the protein of interest is an SR-resident protein. It will be necessary to investigate the effects of acute MG29 and sar ablation to determine their specific contributions to muscle function and to develop possible therapies for muscular dystrophy and for the prevention of fatigue-related decrease in muscle function. Our novel methodology in cultured mammalian muscle fibers should also allow for the differentiation between the
acute (e.g., RNAi) and the compensatory effects of relevant triad junction proteins in muscle function.

GRANTS

This work was supported by National Institutes of Health (NIH) Grants RO1-AG-15556, RO1-HL-69000, RO1-CA-95379, and RO1-DK-51770 to J. Ma; a Robert Wood Johnson Foundation Research Grant, an NIH-National Institute on Aging Faculty Development Grant, and an American Heart Association Scientist Development Grant to M. Brotto; and an American Heart Association Postdoctoral Fellowship to N. Weisleder.

REFERENCES