UC Davis
UC Davis Previously Published Works

Title
Coding sequences of sarcoplasmic reticulum calcium ATPase regulatory peptides and expression of calcium regulatory genes in recurrent exertional rhabdomyolysis

Permalink
https://escholarship.org/uc/item/5rf200pk

Journal
Journal of Veterinary Internal Medicine, 33(2)

ISSN
0891-6640

Authors
Valberg, Stephanie J
Soave, Kaitlin
Williams, Zoë J
et al.

Publication Date
2019-03-01

DOI
10.1111/jvim.15425

Peer reviewed
Coding sequences of sarcoplasmic reticulum calcium ATPase regulatory peptides and expression of calcium regulatory genes in recurrent exertional rhabdomyolysis

Stephanie J. Valberg1 | Kaitlin Soave1 | Zoë J. Williams1 | Sudeep Perumbakkam1 | Melissa Schott1 | Carrie J. Finno2 | Jessica L. Petersen3 | Clara Fenger4 | Joseph M. Autry5 | David D. Thomas5

1McPhail Equine Performance Center, Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, Michigan
2Department of Population Health and Reproduction, University of California-Davis, Davis, California
3Department of Animal Science, University of Nebraska-Lincoln, Lincoln, Nebraska
4Equine Integrated Medicine, PLC, Lexington, Kentucky
5Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota Medical School, Minneapolis, Minnesota

Correspondence
Stephanie J. Valberg, McPhail Equine Performance Center, Department of Large Animal Clinical Sciences, Michigan State University, 736 Wilson Rd, East Lansing, MI 48824.
Email: valbergs@cvm.msu.edu

Funding information
Foundation for the National Institutes of Health, Grant/Award Number: R01HL129814, R37AG26160; Grayson Jockey Club Research Foundation; DDT NIH, Grant/Award Number: R01 HL129814, R37 AG26160; Morris Animal Foundation, Grant/Award Number: D16Eq0040

Background: Sarcolipin (SLN), myoregulin (MRLN), and dwarf open reading frame (DWORF) are transmembrane regulators of the sarcoplasmic reticulum calcium transporting ATPase (SERCA) that we hypothesized played a role in recurrent exertional rhabdomyolysis (RER).

Objectives: Compare coding sequences of SLN, MRLN, DWORF across species and between RER and control horses. Compare expression of muscle Ca2+ regulatory genes between RER and control horses.

Animals: Twenty Thoroughbreds (TB), 5 Standardbreds (STD), 6 Quarter Horses (QH) with RER and 39 breed-matched controls.

Methods: Sanger sequencing of SERCA regulatory genes with comparison of amino acid (AA) sequences among control, RER horses, human, mouse, and rabbit reference genomes. In RER and control gluteal muscle, quantitative real-time polymerase chain reaction of SERCA regulatory peptides, the calcium release channel (RYR1), and its accessory proteins calsequestrin (CASQ1), and calstabin (FKBP1A).

Results: The SLN gene was the highest expressed horse SERCA regulatory gene with a uniquely truncated AA sequence (29 versus 31) versus other species. Coding sequences of SLN, MRLN, and DWORF were identical in RER and control horses. A sex-by-phenotype effect occurred with lower CASQ1 expression in RER males versus control males (P < .001) and RER females (P = .05) and higher FKBP1A (P = .01) expression in RER males versus control males.

Conclusions and Clinical Importance: The SLN gene encodes a uniquely truncated peptide in the horse versus other species. Variants in the coding sequence of SLN, MRLN, or DWORF were not associated with RER. Males with RER have differential gene expression that could reflect adaptations to stabilize RYR1.

KEYWORDS
exercise, myopathy, RYR1, skeletal muscle, tying up

Abbreviations: AA, amino acid; ATP2A1, gene encoding sarcoplasmic reticulum calcium transporting ATPase; BLAT, BLAST-like alignment tool; CASQ1, calsequestrin; cDNA, complementary DNA; CK, creatine kinase; CT, cycle thresholds; DWORF, dwarf open reading frame; ER, exertional rhabdomyolysis; F, female; FKBP1A, calstabin; G, gelding; GAPDH, glyceraldehyde phosphate dehydrogenase; GYS1, glycogen synthase 1; MRLN, myoregulin; PAS, periodic acid-Schiff’s; PCR, polymerase chain reaction; PLN, phospholamban; QH, Quarter Horses; qRT-PCR, quantitative real-time polymerase chain reaction; RER, recurrent exertional rhabdomyolysis; RYR1, calcium release channel; S, stallions; SERCA, sarcoplasmic reticulum calcium transporting ATPase; SLN, sarcolipin; SR, sarcoplasmic reticulum; STD, Standardbred; TB, Thoroughbred; UCSC, University of California, Santa Cruz.

The project was performed at Michigan State University.
1 | INTRODUCTION

Exertional rhabdomyolysis (ER) in horses is characterized by multiple episodes of stiffness, muscle cramping, reluctance to move, and muscle damage and can have many causes.\textsuperscript{1} Exertional rhabdomyolysis affects 5%-7% of Thoroughbred (TB) and Standardbred (STD) racehorses, and recurrence can be so frequent that 17% of ER horses are unable to race again in the same season.\textsuperscript{2-4} The term recurrent exertional rhabdomyolysis (RER) has been used to describe a chronic form of ER in racehorses with a proposed underlying cause of abnormal myoplasmic calcium (Ca\textsuperscript{2+}) regulation.\textsuperscript{5,6} This hypothesis was based on finding a lower threshold for inducing a contracture in isolated skeletal muscle bundles of RER versus control horses exposed to increasing concentrations of halothane, potassium, and caffeine, all of which induce Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR).\textsuperscript{5,6} In addition, higher caffeine-induced Ca\textsuperscript{2+} release was found in cultured myotubes from RER versus control horses, as detected by Fura-2 fluorescence imaging.\textsuperscript{7} Further studies of isolated SR membranes and genetic linkage analysis have not identified an underlying cause for alterations of Ca\textsuperscript{2+} regulation in RER.\textsuperscript{8,9}

Recent discoveries regarding fundamental modes of intracellular Ca\textsuperscript{2+} regulation have identified additional regulatory mechanisms for the SR Ca\textsuperscript{2+} transporting ATPase (SERCA) that may play a role in the genesis of RER in horses. After contraction, SERCA induces muscle relaxation by catalyzing the transport of 2 Ca\textsuperscript{2+} ions into the lumen of the SR using the free energy from hydrolysis of 1 ATP molecule. Phospholamban (PLN) inhibits SERCA activity and is primarily expressed in cardiac and slow twitch muscle fibers.\textsuperscript{10,11} Sarcolipin (SLN), first discovered in 1974 as a peptide that copurifies with SERCA, was subsequently found to decrease the Ca\textsuperscript{2+} affinity of SERCA\textsuperscript{12} and decrease the energetic coupling efficiency of SERCA (Ca\textsuperscript{2+}/ATP transport ratio <2), thereby decreasing SR luminal Ca\textsuperscript{2+} stores.\textsuperscript{12-14} In addition, transcripts that previously were annotated as long noncoding RNAs recently have been found to encode small transmembrane peptides MRLN and dwarf open reading frame (DWORF) that also regulate the activity of SERCA in skeletal muscle (Figure 1).\textsuperscript{15,16} Dwarf open reading frame has been shown to enhance SERCA activity in the mouse heart by displacing PLN and, in cell culture models, by displacing SLN and MRLN (Figure 1). A decrease in SLN and MRLN or increase in DWORF expression could increase SR Ca\textsuperscript{2+} stores by decreased SERCA inhibition (ie, decreased Ca\textsuperscript{2+} affinity), thereby acting to increase calcium release channel (RYR1) Ca\textsuperscript{2+} release and myoplasmic Ca\textsuperscript{2+} concentration during contraction and potentially leading to clinical manifestations of RER.

Our goal was to determine if RER is associated with variants in the coding sequences of SLN, MRLN, or DWORF, altered expression of Ca\textsuperscript{2+} regulatory genes involved in SR Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+} release or both. The first aim of our study was to compare the coding sequences of SLN, MRLN, and DWORF in the horse with other species. The second aim was to determine if the coding sequences for these genes differed between RER and control horses. The third aim was to determine if there was a difference in expression of skeletal muscle Ca\textsuperscript{2+} regulatory genes between horses with and without RER.

![Figure 1](image-url)  
**FIGURE 1** Schematic of key Ca\textsuperscript{2+} regulatory proteins in skeletal muscle sarcomplasmic reticulum (SR). Sarcomplasmic reticulum calcium transporting ATPase (SERCA) is the SR Ca\textsuperscript{2+} pump with isoform SERCA1 expressed in fast twitch type 2 fibers and SERCA2 expressed in slow twitch and cardiac muscle fibers. SERCA is inhibited by sarcolipin (SLN), phospholamban (PLN), or myoregulin (MRLN). Phospholamban primarily inhibits SERCA2, and SLN and MRLN inhibit SERCA1, depending upon species. Dwarf open reading frame (DWORF) displaces the SERCA inhibitors, PLN, SLN, and MRLN. FKBP (calstabin) modulates Ca\textsuperscript{2+} release through the Ca\textsuperscript{2+} release channel which has 2 isoforms: RYR2 (cardiac and slow twitch) and RYR1 (fast twitch muscle fibers). Calsequestrin (CASQ) is the luminal, high-capacity Ca\textsuperscript{2+} binding protein, which directly modulates Ca\textsuperscript{2+} release by RYR. DHPR, the dihydropyridine receptor, is a voltage-gated Ca\textsuperscript{2+} channel that triggers RYR to release Ca\textsuperscript{2+}. The legend (right) indicates the effect of each regulatory protein on myoplasmic Ca\textsuperscript{2+} concentration: increase (+) or decrease (−).

2 | METHODS

2.1 | Pilot study

To determine if SERCA1 (expressed in fast twitch type 2 fibers) or SERCA2 (expressed in cardiac and type 1 muscle fibers) was primarily expressed in equine gluteal muscle, we initially evaluated transcripts per million reads from RNA-seq data obtained from gluteal muscle of 6 healthy Arabian horses (NCBI’s Gene Expression Omnibus GEO Series accession number GSE104388). Mean transcripts per million reads (SD) were 3.7 times higher for SERCA1 (562 ± 152 TPM) than SERCA2 (243 ± 145 TPM). When muscle fiber type composition was assessed for 6 of the TBs in our study, we found they had 50% fewer type 1 fibers than did the Arabian horses used in the RNA-seq analyses (TB: 8% ± 3% type 1, 92% ± 5% type 2; Arabian 17% ± 3% type 1, 83% ± 8% type 2). Thus, SERCA1 rather than SERCA2 seemed to be the primary isoform of interest when studying SERCA inhibitors in TB gluteal muscle.

The study was approved by the Institutional Animal Use and Care Committee of Michigan State University.

2.2 | Comparative amino acid sequences

The entire coding sequences for SLN and MRLN were identified from the annotated references genomes of horse (EquCab2; http://ncbi.nlm.nih.gov/genome/145). Horse coding sequences were verified by comparison to RNA-seq data for Arabian control horses (NCBI’s Gene Expression Omnibus GEO Series accession number GSE104388) and comparison to Sanger sequencing described below. Coding sequences for DWORF were identified in the annotated human and mouse
reference genomes and used as a BLAST-like alignment tool (BLAT) on EquCab2 (University of California, Santa Cruz [UCSC] genome browser; https://genome.ucsc.edu/). Reads for the equine DWORF coding sequence were present in the reference genome, but the reads ended abruptly without a stop codon, suggesting that EquCab2 is not well assembled in the 3’ region of this gene. The RNA-seq data from Arabian horses (GEO Series accession number GSE104388) was used to complete the derived equine DWORF sequence.

The amino acid (AA) sequences of PLN, SLN, and MRLN were compared to the human and mouse sequences because these species have well-established references genomes and to rabbit because we currently are performing comparative biochemical assays on SR preparation from horse and rabbit. The 2016 Ensembl (https://useast.ensembl.org/index.html) was used to evaluate the genome of rabbit (OryrCun; https://www.ncbi.nlm.nih.gov/assembly/GCF_000003625.3), mouse (http://www.informatics.jax.org/), and human (GRCh38.p12 version). For rabbit, coding sequences for DWORF from the annotated human and mouse reference genomes were used to BLAT the respective reference genomes (UCSC genome browser; https://genome.ucsc.edu/).

We also performed comparative sequence analysis for SLN on species closely related to the horse including ass (NCBI accession NW_014638236.1, Genebank ERX607030, ERX607036, ERX607001,) Przewalski’s horse (databases NC_007657973.1, Genebank ABW01057363.1), and zebra as well as another Perisodactyl, the Southern white rhinoceros (http://rohsdb.cmbusc.ucsc.edu/; LOC101603223). To obtain SLN sequence for the zebra (unknown genus), we Sanger sequenced muscle tissue archived in the Neuromuscular Diagnostic Laboratory in the same blinded fashion as described for RER and control horses.

2.3 | Gene sequencing

2.3.1 | Horses

The SLN sequence was determined for 17 TB (11 females [F], 3 geldings [G], 3 stallions [S]), 5 STD (2 F, 2 G, 1 S), and 6 Quarter Horses (QH; 3 F, 3 G) that had experienced repeated episodes of ER and had muscle biopsy specimens archived in the Neuromuscular Diagnostic Laboratory (Supplemental Table 1). Control horses included 13 TB (3 F, 10 G), 6 STD (1 F, 5 G), and 3 QH (1 F, 2 G) with no known history of RER and samples archived in the Neuromuscular Diagnostic Laboratory. The MRLN and DWORF sequences were determined in a subset consisting of 3 TB, 3 STD, and 3 QH with RER and 4 TB, 4 STD, and 3 QH controls (Supplemental Table 1). The RER criteria comprised 2747 bp. For DWORF, based on the open reading frame that begins in exon 1 and encodes the first 4 AA of the protein with the remaining protein being encoded in exon 2, 2 primers were designed to cover 500 bp upstream of the coding sequence, the first 4 AA in exon 1, and exon 2 and the 3’ untranslated region. The region sequenced for DWORF comprised 1300 bp. Primers are listed in Supplemental Table 2.

2.3.2 | DNA isolation and sequencing

Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, Maryland) was used to isolate genomic DNA from hair roots, buffy coat, or frozen muscle samples according to the manufacturer’s protocol.

2.3.3 | Primers

Primers were designed using Primer3Plus software18 to cover the predicted protein coding regions of horse SLN, MRLN, and DWORF as well as the 5’ upstream sequence, likely containing the 5’ untranslated region of the 3 horse genes based on similarities across species. For SLN, 8 primers were designed to cover the possible noncoding exon 1 (2148 bp) and predicted coding exon 2 (2092 bp; Supplemental Table 2). The regions sequenced for SLN comprised 6332 bp. For MRLN, 4 primers were designed to cover noncoding exon 1, possible noncoding exon 2, noncoding exon 3, and coding exon 4 (Supplemental Table 2). The region sequenced for MRLN comprised 2747 bp. For DWORF, the open reading frame that begins in exon 1 and encodes the first 4 AA of the protein with the remaining protein being encoded in exon 2, 2 primers were designed to cover 500 bp upstream of the coding sequence, the first 4 AA in exon 1, and exon 2 and the 3’ untranslated region. The region sequenced for DWORF comprised 1300 bp. Primers are listed in Supplemental Table 2.

2.3.4 | PCR and sequence analysis

Each primer pair was used to amplify intervening genomic DNA using polymerase chain reaction (PCR) in 25 μL reactions that included 2.0 μL sample DNA, 12.5 μL Hot Start PCR 2x Master Mix Taq Polymerase (Thermo Fisher Waltham, MA), 0.5 μL of 20 μM forward and reverse primers (Invitrogen), and 9.5 μL molecular biology grade water. The PCR reactions started with 15 minutes at 95°C, then 35 cycles of 30 seconds at 94°C, 30 seconds at the primer-specific annealing temperature, and 30 seconds at 72°C, followed by a final extension of 10 minutes at 72°C. The PCR products were resolved on 1% agarose gels. The PCR products then were purified using ExoSAP-IT Product Cleanup (Affymetrix, Santa Clara, California) and sequenced by the Michigan State University Research Technology Support Facility Genomics Core using the 96-capillary electrophoretic ABI 2730xl platform (Sanger sequencing). Sequences were aligned to Equcab2.0 (http://ncbi.nlm.nih.gov/genome/145) and analyzed using Sequencher software (version 5.4.5; Gene Codes Corporation).

2.4 | Gene expression

2.4.1 | Horses

Muscle samples were obtained prospectively from 14 fit TB RER racehorses (9 F, 2 G, 3 S; age 5.2 ± 2.4 years) with median serum CK activity of 251 U/L and mean (SD) CK activity of 970 ± 2166 U/L. Samples also were obtained from 20 fit control TB racehorses (9 F, 6 G, 5 S; age 3.4 ± 1.6 years) with median CK activity of 250 U/L and mean CK activity of 267 ± 86 U/L. All horses were housed either at the same race training center or a nearby racetrack in Lexington, Kentucky (Supplemental Table 1). The RER horses had a history of episodes of ER documented by a veterinarian and had not exhibited clinical signs
within 48 hours of muscle biopsy. Control horses were in training and had no history of ER. Twelve of the horses used for gene expression studies also were Sanger sequenced for SLN as described above.

2.4.2 | Primers
Primers for GAPDH, RYR1, SERCA1, CASQ1, DWORF, PLN, MRLN, SLN, and FKBP1A were designed to cross exon-exon boundaries using NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and referencing NCBI EquCab 2.0 (Supplemental Table 3). The glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as a housekeeping control because it showed minimal variability and is less variable across age in skeletal muscle.19

2.4.3 | Muscle biopsies
Gluteus medius muscle biopsy specimens were obtained in the morning 1-4 hours after jogging or light galloping exercise from a standardized site using a modified Bergstrom biopsy needle as previously described.20 A portion of the sample was flash-frozen in liquid nitrogen and stored at −80°C. A second portion was oriented in cross-section and frozen within 12 hours of sampling in isopentane that was suspended in liquid nitrogen.

2.4.4 | Muscle histopathology
Cryostat sections (7-μm thick) were stained with hematoxylin and eosin and PAS and evaluated for the presence of centrally located nuclei, degenerating myofibers, or macrophages.21

2.4.5 | RNA extraction
Total muscle RNA was isolated from flash frozen samples using TRIzol/chloroform extraction after tissue homogenization with a biopulverizer (BioSpec Products, Inc, Fortaleza, Florida) plus RNase-free distilled water. Reactions were run for 40 cycles under the following conditions: denaturation at 95°C for 5 minutes, annealing at 60°C for 1 minute, and 95°C until completion. All reactions then were diluted with sterile nuclease-free distilled water to reach a total volume of 100 μL.

2.4.6 | Complementary DNA synthesis
Complementary DNA (cDNA) was made using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA). Each 20 μL reaction contained 2 μL of 10× RT Buffer, 0.8 μL of 100 mM dNTPs, 2 μL of RT Random Primers, 1 μL of RT Enzyme, approximately 1200 ng of sample RNA, and the remaining volume made up of sterile nuclease-free distilled water. The reactions then were run in a ProFlex PCR system (Applied Biosystems, Life Technologies Waltham, MA) under the following conditions: 25°C for 10 minutes, 37°C for 2 hours, 85°C for 5 minutes, and 4°C until recovery. All reactions then were diluted with sterile nuclease-free distilled water to reach a total volume of 100 μL.

2.4.7 | Quantitative real-time PCR
Genes selected included the Cα2+ release channel (RYR1), calstabin (FKPB1A), which stabilizes Ca2+ leak from RYR1, and calsequestrin (CASQ1), a luminal high-capacity Cα2+ binding protein that modulates RYR1 Ca2+ release. Expression of gene encoding SERCA (ATP2A1), PLN, SLN, MRLN, and DWORF also was determined. Thermocycling for quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using EvaGreen dye (Biotium, Inc, Fremont, California), ROX Reference Dye (Invitrogen, Life Technologies Carlsbad, CA), and Hot Start taq DNA Polymerase (New England BioLabs, Inc Ipswich, MA) using the QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific Waltham, MA). The PCR reactions were run in duplicate (20 μL volume reactions). Each reaction contained 2 μL of sample cDNA, 2 μL of 2.5 mM dNTPs, 2 μL of 10× PCR buffer, 1 μL of EvaGreen dye, 1.5 μL of 1:10 ROX reference dye dilution, 0.125 μL of Hot Start taq DNA Polymerase, 2 μL of 1.6 μM forward primer, 2 μL of 1600 μM reverse primer, and 7.4 μL of sterile nuclease-free distilled water. Reactions were run for 40 cycles under the following conditions: denaturation at 95°C for 10 minutes, annealing at 60°C for 1 minute; melt curve stages at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. Cycle thresholds (CT) were automatically calculated by the QuantStudio 3 Real-Time PCR System. For each gene of interest, 100% geometric efficiency was established. Nontemplate controls run for each gene showed no amplification.

2.5 | Statistical analysis
2.5.1 | Quantitative real-time PCR
Relative quantitation of gene expression was calculated by the comparative threshold cycle method (2−ΔΔCT) using the CT of GAPDH as the reference samples. Each gene expression was compared to its average expression in females and males separately, as described by Livak and Schmittgen.24 Statistical significance was determined by Student’s t-test. A P value of <0.05 was considered statistically significant. Bonferroni correction was used to calculate adjusted P value (Padj). The adjusted P value was calculated as (P * number of genes tested).25

### Table 1: Mean (SD) gene expression relative to GAPDH (ΔCT)

<table>
<thead>
<tr>
<th>Gene</th>
<th>RER females</th>
<th>Control females</th>
<th>RER males</th>
<th>Control males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 9</td>
<td>N = 9</td>
<td>N = 5</td>
<td>N = 11</td>
</tr>
<tr>
<td>RYR1</td>
<td>5.57 ± 0.64a</td>
<td>5.64 ± 0.82a</td>
<td>5.55 ± 0.65a</td>
<td>5.81 ± 0.66a</td>
</tr>
<tr>
<td>FKBP1A</td>
<td>5.19 ± 2.76a</td>
<td>6.36 ± 1.32a</td>
<td>4.31 ± 1.49ab</td>
<td>6.77 ± 0.67ab</td>
</tr>
<tr>
<td>CASQ1</td>
<td>−2.31 ± 2.14ac</td>
<td>−3.32 ± 1.83a</td>
<td>−0.23 ± 1.42bc</td>
<td>−1.64 ± 1.43bc</td>
</tr>
<tr>
<td>ATP2A1</td>
<td>7.98 ± 1.03a</td>
<td>8.06 ± 0.55a</td>
<td>7.02 ± 1.99a</td>
<td>8.72 ± 1.51a</td>
</tr>
<tr>
<td>SLN</td>
<td>−4.67 ± 1.74a</td>
<td>−5.31 ± 1.68a</td>
<td>−3.23 ± 1.24a</td>
<td>−4.18 ± 2.34a</td>
</tr>
<tr>
<td>MRLN</td>
<td>6.91 ± 0.69a</td>
<td>7.55 ± 1.20a</td>
<td>7.52 ± 0.91a</td>
<td>6.21 ± 1.16a</td>
</tr>
<tr>
<td>PLN</td>
<td>8.27 ± 1.48a</td>
<td>8.12 ± 1.61a</td>
<td>8.42 ± 1.68a</td>
<td>8.53 ± 1.34a</td>
</tr>
<tr>
<td>DWORF</td>
<td>7.95 ± 1.41a</td>
<td>7.94 ± 1.07a</td>
<td>7.64 ± 1.14a</td>
<td>8.64 ± 1.61a</td>
</tr>
</tbody>
</table>

Genes include RYR1 and its regulators FKBP1A and CASQ1 as well as SERCA1 (ATP2A1) and its inhibitors SLN, PLN, and MRLN, plus DWORF which displaces the SERCA inhibitors. Different letters indicate differences between rows. P ≤ .05. Abbreviations: ATP2A1, gene encoding sarcoplasmic reticulum calcium transporting ATPase; CASQ1, calsequestrin; CT, cycle thresholds; DWORF, dwarf open reading frame; FKBP1A, calstabin; GAPDH, glyceraldehyde phosphate dehydrogenase; MRLN, myoregulin; PLN, phospholamban; RER, recurrent exertional rhabdomyolysis; RYR1, calcium release channel; SERCA, sarcoplasmic reticulum calcium transporting ATPase; SLN, sarcoplamin.
(Table 1). Data was tested for normality using the Shapiro Wilks test. A 2-way analysis of variance and Tukey post hoc test were performed to examine differences in ΔCT values for RER and control horses stratified by sex using GraphPad Prism 7 (Graphpad Software, La Jolla, California).

3 | RESULTS

3.1 | Comparison of AA sequences

3.1.1 | Sarcolipin

The coding sequence of $SLN$ in RER and control horses was truncated at 29 versus 31 AA relative to the human, mouse, rabbit, and Southern white rhinoceros sequences. The zebra, ass, and Przewalski’s horse had the same truncated sequence as did the horse (Supplemental Table 4). Homology of the horse $SLN$ AA sequence was 77% to rabbit (24/31), 77% to human (24/31), and 81% to mouse (25/31). Most importantly, the $SLN$ AA sequence was missing putative regulatory sites Ser4, Thr5, Cys9, and Tyr31 (Figure 2).

3.1.2 | Myoregulin

The myoregulin ($MRLN$) AA sequence was similar in length across species at 46 AA. Sequence identity of horse $MRLN$ was 78% with rabbit (36/46), 85% with human (39/46), and 74% with mouse (34/46). Most AA differences occurred in the cytoplasmic domain with only 1 AA impacting charge, horse neutral Thr15 versus human and rabbit basic Lys15.

3.1.3 | Dwarf open reading frame

The $DWORF$ peptide was similar in length between horse and human at 35 AA and was 34 AA in mouse with 3 AA that could not be deduced from the rabbit reference genome (Figure 2). Sequence homology of horse $DWORF$ was 86% with human (30/35) and 69% with mouse (24/35). Amino acid substitutions that altered AA charge were not identified when comparing AA across horse, human, and mouse.

3.2 | Coding sequence of $SLN$, $MRLN$, $DWORF$ in RER and control horses

No differences were detected in coding sequences of $SLN$, $MRLN$, and $DWORF$ between RER and control horses.

3.3 | Gene expression

Sarcolipin was the most highly expressed SERCA regulatory gene (Table 1). No significant difference was found in the expression level of Ca$^{2+}$ regulatory genes between control females and control males or between RER females and control females (Figure 3). An impact of sex and phenotype was observed in which RER males had significantly higher expression of $FKBP1A$ ($P = .01$) than did control males (Figure 3). The RER males had lower expression of $CASQ1$ than did control females ($P < .001$) and lower expression than RER females ($P = .05$; Figure 3).

![FIGURE 2](image) Amino acid sequence (AA) of $SLN$, $PLN$, $MRLN$, and $DWORF$ derived from mouse, human, and rabbit reference genomes, and Sanger sequencing of horse genes in the present study. Yellow highlight indicates AA substitution or deletion unique to horse. Green highlight indicates highly conserved residues near the myoplasmic-membrane interface of SERCA inhibitory peptides. Asterisk (*) indicates regulatory site residues. Question mark (?) indicates putative phosphorylation sites or lack of consensus sequence for rabbit $DWORF$ from the reference genome. $DWORF$, dwarf open reading frame; $MRLN$, myoregulin; $PLN$, phospholamban; SERCA, sarcoplasmic reticulum calcium transporting ATPase; $SLN$, sarcolipin.
FIGURE 3  Quantitative real-time polymerase chain reaction for genes involved in myoplasmic Ca\(^{2+}\) regulation in RER and control horses stratified by sex and expressed as relative abundance of transcripts compared to GAPDH and their respective control group (\(\Delta\Delta C_T\)). Genes include RYR1 and its regulators FKBP1A and CASQ1 as well as SERCA (ATP2A1) and its inhibitors SLN, PLN, and MRLN, plus DWORF which displaces the SERCA inhibitors. Asterisks indicate difference for FKBP1A at \(P = .01\) and CASQ1 at \(P = .05\). ATP2A1, gene encoding sarcoplasmic reticulum calcium transporting ATPase; CASQ1, calsequestrin; DWORF, dwarf open reading frame; FKBP1A, calstabin; GAPDH, glyceraldehyde phosphate dehydrogenase; MRLN, myoregulin; PLN, phospholamban; RER, recurrent exertional rhabdomyolysis; RYR1, calcium release channel; SERCA, sarcoplasmic reticulum calcium transporting ATPase; SLN, sarcolinip

4 | DISCUSSION

In resting striated muscle across all species, Ca\(^{2+}\) is tightly regulated to maintain 10 000-fold lower myoplasmic Ca\(^{2+}\) than SR luminal Ca\(^{2+}\) concentration because of the activity of SERCA.\(^{36}\) The activity of SERCA is regulated by myoplasmic Ca\(^{2+}\) and by the inhibitory peptides, PLN and SLN.\(^{37}\) More recently, MRLN in mouse skeletal muscle and sarcolamban in fly heart have been reported to be important SERCA inhibitors that have highly conserved protein sequences and molecular structure across species.\(^{16,38,39}\) Such conservation for over 550 million years has been present for millions of years, before the divergence of Equus caballus, Equus przewalski, Equus grevi, and Equus asinus, and suggests a unique mechanism for myoplasmic Ca\(^{2+}\) regulation in the horse.\(^{44}\)

A selection advantage for RER is suggested by the fact that STD horses with RER have faster racing times from a standing start than do STD without RER and by the high prevalence of RER in STD and TB at 5%-7%.\(^{2,3}\) Small increases in myoplasmic Ca\(^{2+}\) concentration during muscle relaxation induced by the unique protein sequence of equine SLN could provide a selection advantage to horses with their superior athletic capacity by facilitating Ca\(^{2+}\) entry into mitochondria (which activates ATP production and metabolic processes), activating Ca\(^{2+}\)-dependent signaling pathways important for programming an oxidative muscle phenotype, and increasing the power of muscle contraction by initial enhancement of actomyosin force production.\(^{35}\) Whereas slight increases in myoplasmic Ca\(^{2+}\) could enhance speed and endurance, excessive increases in myoplasmic Ca\(^{2+}\) lead to persistent myofiber contracture, enhanced reactive oxygen production, activation of proteases, and myodegeneration.\(^{46}\) Thus, in horses, it is possible that slight alterations in the regulation of myoplasmic Ca\(^{2+}\) result in a fine balance between enhanced speed on the 1 hand and RER on the other.

The distinctive sequence of equine SLN made it a potential candidate gene for RER. However, no differences in SLN coding sequence were detected among 18 TB, 5 STD, and 6 QH with RER versus control horses. Coding sequences of MRLN and its inhibitor DWORF also were evaluated in a small number of horses, but no mutation associated with RER was identified. Additional resources were not directed to sequence more horses for MRLN and DWORF, because those RER horses that were sequenced had repeated episodes of ER of sufficient concern to submit muscle biopsy samples but no mutations were found. Multiple causes for RER may exist, some of which may be breed specific, and individual horses may have mutations in genes that were not evaluated in our study. However, based on our results, we propose that there is no high-frequency coding mutation in SERCA inhibitors SLN or MRLN or the dominant-negative SERCA activator DWORF in TB, STD, and QH with RER. Sequencing of PLN was not performed on the horses in our study because PLN had much lower mRNA expression in skeletal muscle than did SLN and the reference genomes across species had identical PLN AA sequences. Our results did not eliminate a PLN mutation as a basis for RER. Previous studies of RER have failed to identify a single genome-wide significant candidate locus for RER, suggesting that multiple genes, strong environmental influences, or both are at play.\(^{47,48}\) The heritability of RER in TB and STD horses has been estimated at approximately 0.40.\(^{49}\) Environmental factors such as sex, diet, fitness, and stress also have been shown to play important roles in expression of RER.\(^{3,49}\)

A previous study of gene expression of RER in gluteal muscle of 4 female and 1 male French STD and 6 male and 4 female control
STD horses was performed using a mouse-equine microarray.\(^5\) In contrast to the present study, muscle biopsy specimens were taken within 24 hours of an episode of ER. The previous study found that gene transcripts involved in muscle fiber Ca\(^{2+}\) homeostasis were modulated in a way that could increase myoplasmic Ca\(^{2+}\) concentration with down-regulation of ATP2A1, RYR1, and several other genes impacting myoplasmic, SR, or mitochondrial Ca\(^{2+}\) load (SLC8A1, UCP2, ANXA6). The SLN and CASQ1 genes were not included in the array, and ATP2A1 was found to be upregulated in the same samples using qRT-PCR. These results are difficult to compare with our study especially because they were not stratified according to sex and because the 2 studies sampled RER muscle at different times: between episodes versus within 1 day of an ER episode.

A sex bias exists for the expression of RER with males being less prone to RER than females.\(^3,4\) It is noteworthy that sex-specific differences in Ca\(^{2+}\) regulatory gene expression were found in RER horses in our study. For example, RER males had lower expression of CASQ1 than did RER females \((P = .05)\) and control females \((P < .001)\). A sex effect of CASQ1 expression and muscle disease is seen in murine models in which male CASQ1-null mice developed fatal stress-induced malignant hyperthermia-like reactions, whereas female CASQ1-null mice were protected.\(^5,1,2\) Calsequestrin is integral in regulating RYR1 and excitation-contraction coupling.\(^5\) It was not possible in our study to standardize the time interval between an episode of ER and when the muscle biopsy specimen was obtained for gene expression analysis. We therefore selected horses with modest increases in CK activity (median, 251 U/L; maximum, 8453 U/L) to prevent muscle cell damage from being the primary driver of altered gene expression. A weakness of our study was that SLN gene expression was evaluated without measurement of SLN protein expression. Examination of SLN protein content presented challenges because commercially available antibodies to SLN did not recognize the unique sequence of equine SLN (personal observation). Further experiments using a customized anti-horse-SLN antibody would be necessary to correlate SLN gene and protein expression. The notable RER sex-specific difference in CASQ1 and FKBP1A expression was an unexpected finding in our study, and further experiments are needed to examine their impact on RER in males and females.

In conclusion, our results show that the Equus species has a novel SLN AA sequence with the potential for unique regulation of the Ca\(^{2+}\) affinity of SERCA. In the horses studied, mutations in the coding sequences of SLN, MRLN, or DWORF were not identified in TB, STD, or QH horses with RER. Differential expression of RYR1 regulators FKBP1A and CASQ1 in RER males suggests that genesis of RER could be impacted by a sex-specific alteration in myoplasmic Ca\(^{2+}\) regulation.
REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.