SR Ca\(^{2+}\) leak in skeletal muscle fibers acts as an intracellular signal to increase fatigue resistance

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Effective practices to improve skeletal muscle fatigue resistance are crucial for athletes as well as patients with dysfunctional muscles. To this end, it is important to identify the cellular signaling pathway that triggers mitochondrial biogenesis and thereby increases oxidative capacity and fatigue resistance in skeletal muscle fibers. Here, we test the hypothesis that the stress induced in skeletal muscle fibers by endurance exercise causes a reduction in the association of FK506-binding protein 12 (FKBP12) with ryanodine receptor 1 (RYR1). This will result in a mild Ca\(^{2+}\) leak from the sarcoplasmic reticulum (SR), which could trigger mitochondrial biogenesis and improved fatigue resistance. After giving mice access to an in-cage running wheel for three weeks, we observed decreased FKBP12 association to RYR1, increased baseline [Ca\(^{2+}\)], and signaling associated with greater mitochondrial biogenesis in muscle, including PGC1α1. After six weeks of voluntary running, FKBP12 association is normalized, baseline [Ca\(^{2+}\)] returned to values below that of nonrunning controls, and signaling for increased mitochondrial biogenesis was no longer present. The adaptations toward improved endurance exercise performance that were observed with training could be mimicked by pharmacological agents that destabilize RYR1 and thereby induce a modest Ca\(^{2+}\) leak. We conclude that a mild RYR1 SR Ca\(^{2+}\) leak is a key trigger for the signaling pathway that increases muscle fatigue resistance.

Introduction

Ca\(^{2+}\) plays a central role in intracellular signaling in most cell types. Ca\(^{2+}\) plays an essential role in initiating contraction in skeletal muscle. The neural activation triggers action potentials that propagate along the surface membrane of muscle fibers and into the transverse tubular system, where they activate voltage sensors, the dihydropyridine receptors (DHPRs). Activated DHPRs open the SR Ca\(^{2+}\) release channel, ryanodine receptor 1 (RYR1), resulting in an increase in the free cytosolic [Ca\(^{2+}\)] ([Ca\(^{2+}\)]). Increasing [Ca\(^{2+}\)] exposes the myosin-binding site on the actin filament, leading to repetitive cycles of myosin head (cross-bridge) binding and muscle contraction (Gordon et al., 2000; Allen et al., 2008). Cross-bridge cycles and the continuous pumping of Ca\(^{2+}\) back into the SR during contractions are highly energy-demanding processes. Thus, oxidative capacity is a key determinant of muscle endurance, i.e., the ability to maintain contractile function during repeated cycles of SR Ca\(^{2+}\) releases and contractions (Holloszy et al., 1970; Holloszy and Coyle, 1984; Allen et al., 2008; Ekblom-Bak et al., 2014; Cheng et al., 2018).

The large RYR1 tetramer (~2 megadalton) is the core of a protein complex, which includes the FK506-binding protein 12 (FKBP12, also known as calstabin1; Ahern et al., 1994; Zalk et al., 2015). If FKBP12 is dissociated from RYR1, the channel has an increased open probability, i.e., it becomes “leaky” (Ahern et al., 1997). Prolonged periods of stress may lead to hyperphosphorylation of RYR1, causing a severe SR Ca\(^{2+}\) leak due to disassociation of FKBP12 (Marx et al., 2000; Aydin et al., 2008). Modifications of RYR1 induced by reactive oxygen/nitrogen species also causes severe disassociation of FKBP12 and subsequent SR Ca\(^{2+}\) leakage (Bellinger et al., 2008). Severe FKBP12 disassociation from RYR1 is linked to muscle weakness in overtraining (Bellinger et al., 2008), as well as various diseases, such as muscle dystrophy (Bellinger et al., 2009), breast cancer–related cachexia (Wan et al., 2015), and ventilator-induced diaphragmatic dysfunction (Matecki et al., 2015). However, increased SR Ca\(^{2+}\) leak leading to increased [Ca\(^{2+}\)], at rest may not always be deleterious. A minor elevation of baseline [Ca\(^{2+}\)], which in itself is not sufficient to initiate contraction, may act as a signal for increased mitochondrial biogenesis by activating Ca\(^{2+}\)-sensing proteins, such as Ca\(^{2+}\)-calmodulin–dependent protein kinase II, calcineurin, or cAMP response element–binding protein, resulting in transcription of proteins important for oxidative capacity (Ojuka et al., 2002; Wu et al., 2002; Tavi and Westerblad, 2011; Bruno et al., 2014). Furthermore, a prolonged increase in [Ca\(^{2+}\)], can increase the expression of peroxisome
proliferator–activated receptor γ coactivator-1α (PGC1α; Wu et al., 2002; Ojuka et al., 2003; Wright et al., 2007), which is tightly associated with mitochondrial numbers, volume and protein content (Wu et al., 1999; Lehman et al., 2000; Baar et al., 2002), and improved exercise endurance in humans (Pilegaard et al., 2003; Norrbom et al., 2004).

Endurance exercise is the most effective method to increase oxidative capacity and mitochondrial content in skeletal muscle (Holloszy and Coyle, 1984; Booth and Thomason, 1991). Nevertheless, the immediate cellular triggers promoting increased oxidative capacity in response to endurance training are not fully understood. The present study is based on the hypothesis that the stress induced in skeletal muscle fibers by endurance exercise causes decreased FKBP12 association to RYR1 and SR Ca2+ leak, which trigger mitochondrial biogenesis, leading to improved fatigue resistance. The hypothesis was tested by investigating RYR1 modifications, cellular Ca2+ handling, mitochondrial biogenesis, and fatigue resistance in muscles from mice either engaged in voluntary running or exposed to pharmacological agents that induce SR Ca2+ leak.

### Materials and methods

#### Ethical approval

Animal experiments complied with the Swedish Animal Welfare Act, the Swedish Welfare Ordinance, and applicable regulations and recommendations from Swedish authorities. The study was approved by the Stockholm North Ethical Committee on Animal Experiments (no. N120/13). We used a total of 81 male C57BL/6N (Harlan) mice with an age of 8 wk at the start of interventions. Animals were housed in a temperature-controlled environment with a 12-h light–dark cycle and were provided with standard rodent chow and water ad libitum. Mice were euthanized by rapid cervical dislocation before tissue extraction.

#### Mouse experiments

Mice were individually housed in cages equipped with a wireless low profile running wheel (ENV-044; Med Associates), and running distance was continuously measured for up to 6 wk. Sedentary controls were similarly housed with locked running wheels. Pharmacologically treated mice were housed in standard cages and injected intraperitoneally every 48 h for 3 wk with 0.7% sterile NaCl solution containing 0.1% DMSO with or without 10 ng/g body weight rapamycin (Sigma-Aldrich) or synthetic ligand of FKBP12 (SLF; Cayman Chemicals). These drugs destabilize RYR1, and the concentration of rapamycin and SLF used here has previously been shown not to have any measurable mTOR (mammalian target of rapamycin)-mediated effects (Lee et al., 2014).
In vivo endurance test

An exhaustion test was conducted after 3 wk on sedentary and voluntary running mice and mice injected with rapamycin or SLF. Endurance was assessed using a mouse treadmill (Exer 3/6; Columbus Instruments). Mice were prepared for the exhaustion test by running on the treadmill for 10 min/d for 4 d before the test. During the test, mice ran at 25° uphill, and an initial 10-min warm-up at a speed of 10 m/min was followed by gradual speed increases of 2 m/min every 2 min. Endurance was scored as the speed during the last interval completed before exhaustion. Exhaustion was determined as the time when the mouse withstood three mild electric shocks (0.1 mA, 2 Hz) without attempts to continue running. This exhaustion test provides an indirect measure of mouse maximal oxygen uptake (VO₂max) (Kemi et al., 2002).

Force and [Ca²⁺], measurements in single muscle fibers

Intact, single muscle fibers were dissected from flexor digitorum brevis (FDB) muscles as described elsewhere (Cheng and Westerblad, 2017). The isolated fiber was mounted in a stimulation chamber at optimum length and superfused with Tyrode's solution (in mM): NaCl, 121; KCl, 5.0; CaCl₂, 1.8; MgCl₂, 0.5; NaH₂PO₄, 0.4; NaHCO₃, 24.0; EDTA, 0.1; glucose, 5.5. Fetal calf serum (0.2%) was added to the solution to improve muscle fiber survival. The solution was bubbled with 5% CO₂–95% O₂, which gives an extracellular pH of 7.4. Experiments were performed at room temperature (±25°C). Tetanic stimulation was achieved by supramaximal current pulses (duration, 0.5 ms) delivered via platinum plate electrodes lying parallel to the muscle fiber.

The fluorescent Ca²⁺ indicator indo-1 (Invitrogen/Molecular Probes) was microinjected into the isolated fiber. The fiber was allowed to rest for at least 30 min after being injected with indo-1. It was then stimulated by individual 350-ms stimulation trains at 10 to 150 Hz given at 1-min intervals. Indo-1 was excited at 360 nm, and emitted fluorescence was measured at 405 nm and 495 nm. The fluorescence ratio of indo-1 was converted to [Ca²⁺], using an intracellularly established calibration curve (Andrade et al., 1998). Tetanic [Ca²⁺], was measured as the mean indo-1 fluorescence during tetanic stimulation trains and basal [Ca²⁺], as the mean over ~200 ms before stimulation. Tetanic force was measured as the mean over 100 ms where force was maximal. Fatigue was induced by repeated tetanic stimulations (70 Hz, 350-ms duration) given at 2-s intervals for 100 contractions.

Measurements of cellular Ca²⁺ entry

Mn²⁺ quenching of fura-2 fluorescence (Hopf et al., 1996) was used to assess cellular Ca²⁺ entry in FDB fibers of sedentary control mice and of mice performing 3 or 6 wk voluntary running. Mouse FDB muscles were isolated and incubated in DMEM (Sigma-Aldrich) with 10% fetal calf serum and 0.3% type I collagenase (Sigma-Aldrich) for 2 h at 37°C. Dissociated single muscle fibers were plated on laminin coated glass bottom dishes (Mattek) in DMEM with 10% fetal calf serum and incubated for at least 1 h at 37°C. Fibers were loaded with 4 ng/µl fura-2 AM (Invitrogen/Molecular Probes) for 30 min and then washed with Tyrode’s for 30 min. Fura-2 was excited at 360 nm (i.e., the isosbestic point where fluorescence is independent of Ca²⁺), and emitted fluorescence was measured at 495 nm. Baseline fluorescence was measured for 50 s, after which 1 mM MnCl₂ was added and the signal was followed for 50 s. Fibers were then stimulated with 50 repeated 70-Hz, 350-ms tetani given at 2-s intervals, and the fluorescent signal was followed for 500 s subsequent to stimulation, after which MnCl₂ was washed out. The slope of fluorescence decay was measured for 50 s before and between 100 and 400 s after stimulation and expressed relative to the fluorescent signal at the start of the respective measurement period.

Western blotting and immunoprecipitation (IP)

Mouse tibialis anterior muscles were homogenized with a ground glass homogenizer in ice-cold homogenization buffer (20 µl per mg wet weight, pH 7.6) consisting of (in mM): HEPES, 20; NaCl, 150; EDTA, 5; KF, 25; Na₃VO₄, 1; and 20% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail (Roche; 1 tablet/50 ml). The homogenate was centrifuged at 700 g for 10 min at 4°C. Protein content of the supernatant was determined using the Bradford assay (#500-0006; Bio-Rad) and frozen at −80°C until protein analysis. On the day of gel electrophoresis, samples were diluted 1:1 in Laemmli buffer (Bio-Rad) with 5% 2-mercaptoethanol and heated to 95°C for 5 min. For IP, 1 µg anti-RYR1 (ab2868, Abcam) was bound to 12 µl G-protein Dynal magnetic beads (Invitrogen) per the manufacturer’s instructions. Samples were plated on laminin coated glass bottom dishes (Mattek) in DMEM with 10% fetal calf serum and incubated for at least 1 h at 37°C. Fibers were loaded with 4 ng/µl fura-2 AM (Invitrogen/Molecular Probes) for 30 min and then washed with Tyrode’s for 30 min. Fura-2 was excited at 360 nm (i.e., the isosbestic point where fluorescence is independent of Ca²⁺), and emitted fluorescence was measured at 495 nm. Baseline fluorescence was measured for 50 s, after which 1 mM MnCl₂ was added and the signal was followed for 50 s. Fibers were then stimulated with 50 repeated 70-Hz, 350-ms tetani given at 2-s intervals, and the fluorescent signal was followed for 500 s subsequent to stimulation, after which MnCl₂ was washed out. The slope of fluorescence decay was measured for 50 s before and between 100 and 400 s after stimulation and expressed relative to the fluorescent signal at the start of the respective measurement period.

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Voluntary running enhances SR Ca\(^{2+}\) pumping and decreases store-operated Ca\(^{2+}\) entry. (A) Original Western blots (top) and boxplots of relative expression (below) show increased SERCA1 and decreased STIM1 protein expression in tibialis anterior muscles of 3-wk and 6-wk running mice with actin as loading control; data are presented relative to the mean pixel density in controls, which was set to 100% (n = 8, 4, and 4 for control, 3 wk, and 6 wk, respectively). (B) Mn\(^{2+}\) quenching of fura-2 fluorescence shows decreased rate of Ca\(^{2+}\) entry after repeated contractions in FDB fibers from 6-wk running mice. Representative fluorescent signal traces (above) over time obtained before and after stimulating the isolated fibers to produce repeated tetanic contractions. Boxplots comparisons of the slope (below) of fura-2 fluorescence decay before (left) and after (right) repeated tetanic contractions (n = 13–14 fibers from eight, four, and four mice for control, 3 wk, and 6 wk, respectively). *, P < 0.05; **, P < 0.01; ***, P < 0.001, 3 wk or 6 wk vs. sedentary controls (Ctrl) with one-way ANOVA. Box plots show median, quartiles, and min/max.

Figure 3.

Enzymatic activity
Mouse tibialis anterior muscles were homogenized in ice-cold buffer (pH 7.4; 50 µl/mg wet weight) consisting of (in mM): Tris, 50; sodium citrate, 5; MnCl\(_2\), 0.6; cysteine, 1; and 0.05% (vol/vol) Triton X-100, pH 7.4. The homogenate was centrifuged for 1 min at 1,400 g (4°C) and aliquots of the supernatant were frozen at –80°C until citrate synthase activity assay. Citrate synthase activities were analyzed with standard spectrophotometric method based on generation of free coenzyme A reacting with 5,5′-dithio-bis-[2-nitrobenzoic acid] (DTNB; Bass et al., 1969). The activities were measured at room temperature under conditions that yielded linearity with respect to extract volume and time. The supernatant protein content was determined using the Bradford assay (BioRad), and activities were adjusted for protein content.

Messenger RNA (mRNA) expression
Total RNA was obtained from mouse extensor digitorum longus by mechanical homogenization using 20× RNeasy lysis buffer (QIAGEN) with 1% β-mercaptoethanol. Homogenates were diluted in 1 ml Trizol (Invitrogen) and mRNA was purified via centrifugation. The extracted mRNA was treated with DNase (Ambion; Invitrogen), and 500 ng complementary DNA (cDNA) was prepared using reverse transcript-PCR (SuperScript III First-Strand Synthesis RT-PCR; Invitrogen). The cDNA was frozen at –80°C until the day of quantitative PCR. Final cDNA was diluted to 2.5 ng/µl, and real-time determination of transcript abundance was performed with SYBR-Green–based real-time quantitative PCR (BioRad) using 1.5 µl cDNA solution per reaction (i.e., ~3.75 ng cDNA) in 5 µl final volume. Table 1 provides the sequences of primers used. Each gene is calculated as relative to the transcript abundance of the housekeeping gene hypoxanthine guanine phosphoribosyltransferase (Hprt1).

50 µl Laemmli buffer (Bio-Rad) with 5% 2-mercaptoethanol and heated to 95°C for 5 min. For the gel electrophoresis, 10 µg of protein or 15 µl IP eluate was run on a 4–12% precast Bis–Tris gel (NP0336PK2, NuPAGE; Invitrogen) and transferred onto polyvinylidine fluoride membranes (Immobilon FL; Millipore, Billerica). Membranes were then blocked with Li-Cor blocking buffer (LI-COR Biosciences) and the band density of actin was used as loading control. Actin and RYR1 were used as loading control for the Western blot and IP experiments, respectively. Equal protein loading to each lane was also verified with protein staining of membranes (#161-0436; Bio-Rad). For S6K1 signaling, membrane was first stained with rabbit anti-S6K1 antibody [E343] (ab32529; Abcam) and reblotted with IRDye 800–conjugated donkey anti-rabbit IgG (926-68072, 926-32213; LI-COR) and subsequently scanned. Membrane was then stripped using Restore Fluorescent Western Blot Stripping Buffer (ThermoFisher), per the manufacturer’s instructions. Membrane was relocked and blotted with rabbit anti-S6K1 antibody [E343] (ab32529; Abcam) and reblotted with IRDye 800–conjugated donkey anti-rabbit IgG (926-68072, 926-32213; LI-COR). Signal is calculated as relative S6K1 (phospho-T412) to total S6K1.

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transferrase (HPRT), which did not differ between groups. Data are presented as relative expression compared with average control, which was set to 1.0.

Statistical analyses

Data are presented as mean ± SEM or as boxplots showing median, quartiles, and min/max. Statistical significant changes were assessed using a two-tailed paired or unpaired Student’s t test or one-way ANOVA with Holm–Sidak post hoc test when comparing more than two groups. Significant differences were set as P < 0.05. All statistical analyses were performed with the SigmaPlot 13.0 software for Windows (Systat Software).

Results

Increasing voluntary running causes RYR1 destabilization and increased baseline [Ca2+]i, and triggers signaling for mitochondrial biogenesis

Mice given access to an in-cage running wheel performed prolonged low-intensity exercise ~12 h a day and gradually increased their voluntary running distance up to ~15 km per day during the first 3 wk and thereafter the daily running distance remained stable (Fig. 1A). After 3 wk of running, there was an increased SR Ca2+ leak as judged from a modest dissociation of FKBP12 from RYR1 (Fig. 1B), accompanied by a ~25% increased baseline [Ca2+]i, in isolated FDB fibers (Fig. 1C). Conversely, after 6 wk, when the running distance had reached a steady state, FKBP12’s association with RYR1 had returned to the control level (Fig. 1B), and baseline [Ca2+]i was actually ~50% lower than in FDB fibers of nonrunning controls (Fig. 1C). Previous studies have linked exercise and cold exposure–induced leaky RYR1 to hyperphosphorylation likely due to adrenergic activation of protein kinase A, which phosphorylates S2843 on RYR1 (Aydin et al., 2008; Bellinger et al., 2008). We observed an increase in average RYR1 phosphorylation after 3 wk of running, but this increase did not reach statistical significance (P = 0.22; Fig. 1D). Thus, further experiments are required to reveal the mechanisms underlying the FKBP12 dissociation from RYR1 and the resulting increase in baseline [Ca2+]i after 3 wk of increasing running, but this is outside the scope of the present study.

After 3 wk of running, there was a marked increase in skeletal muscle mRNA expression of the mitochondrial biogenesis promoting genes of PGC1α, myocyte enhancer factor-2c (MEF2c), and mitochondrial transcription factor A (TFAM; Fig. 2A), as well as the protein level of PGC1α1 (Fig. 2B). Intriguingly, all of these mitochondria-promoting factors had returned to the control level after 6 wk of running, i.e., they followed the same temporal pattern as the FKBP12 dissociation from RYR1. However, the mRNA expression of cytochrome c oxidase subunit 5B (COX5B) and the protein content of the mitochondrial DNA (mtDNA)-encoded cytochrome c oxidase subunit 1 (COX1), which reflects the mitochondrial volume in muscle, were increased after 3 wk of running and remained high at 6 wk of running (Fig. 2, A and B). Thus, with continued running, the mitochondrial content was maintained at a higher level than in control despite no concomitant up-regulation of mitochondria-promoting factors, which indicates temporal and quantitative differences in the signaling for the initial increase in mitochondrial biogenesis and subsequent maintenance at a higher level.

The decreased [Ca2+]i at rest after 6 wk of running was unexpected and experiments were performed to reveal underlying mechanisms. In addition to RYR1 resting conductance, the rates of active reuptake of Ca2+ into the SR by the SR Ca2+-ATPase (SERCA1) and Ca2+ entering the cell via store-operated Ca2+ entry (SOCE) can affect [Ca2+]i, at rest. Compared with control muscles, the protein expression of SERCA1 was increased by ~70% and ~120% after 3 and 6 wk of running, whereas the SR Ca2+ sensor that activates SOCE, the stromal-interacting molecule 1 (STIM1; Roos et al., 2005; Wei-LaPierre et al., 2013), showed a progressive decrease by ~20% and ~40% (Fig. 3A). We also used Mn2+...
The rate of Mn²⁺ quenching was low in fully rested FDB muscle fibers from controls and runners in the early fast phase after a 150-Hz tetanus showed no notable difference between fibers of 3- and 6-wk runners, respectively (Fig. 5 B). The late slow phase of [Ca²⁺]i can be used to assess changes in SR Ca²⁺ pumping and SR Ca²⁺ leak (Klein et al., 1991; Westerblad and Allen, 1993). To facilitate the comparison of L between groups, N was set to four for all fibers, and values for L and A in each fiber were obtained when d[Ca²⁺]/dt − A[Ca²⁺]⁴ + L = 0. (E and F) Boxplots of baseline [Ca²⁺]i in dissected single FDB fibers from NaCl- and rapamycin-injected mice (n = 7 vs. 5, from four mice). * P < 0.05 Rap or SLF vs. controls (NaCl) with one-way ANOVA. Box plots show median, quartiles, and min/max.

The decay of [Ca²⁺]i after tetanic stimulation can be used to broadly distinguish between effects due to alterations in SR Ca²⁺ pumping or passive SR Ca²⁺ leak. Averaged decays of [Ca²⁺]i following a 150-Hz contractions (70 Hz, 350 ms given at 2-s intervals; Fig. 4, B and C). From these fits, the relation between the rate of [Ca²⁺]i decline (d[Ca²⁺]/dt) and [Ca²⁺]i was fitted to the following equation: d[Ca²⁺]/dt = A[Ca²⁺]⁴ + L, where N = 4.03 ± 0.24 Ca²⁺ binding per SR pump unit (Klein et al., 1991). N = 4. The slow phase of average decays levels off at higher [Ca²⁺]i in fibers of 3-wk runners and rapamycin-injected mice and at lower [Ca²⁺]i in 6-wk runners. (C) Typical example of [Ca²⁺]i decay in Ctrl fiber with least-square curve fit (black line). (D) Data points of the relation between the rate of decline d[Ca²⁺]/dt and [Ca²⁺]i were fitted to the following equation: d[Ca²⁺]/dt = A[Ca²⁺]⁴ − L − 4L, where N was set to 4, for each individual fiber. Values for L and A in each fiber was extrapolated when d[Ca²⁺]/dt − A[Ca²⁺]⁴ + L = 0. Box plots (right) show no difference in tetanic force or tetanic [Ca²⁺]i compared to what others have reported (Klein et al., 1991; Westerblad and Allen, 1993). For this assessment, we first fitted the slow [Ca²⁺]i decay of each individual fiber to the sum of two exponential functions (Fig. 5 C). From these fits, the relation between the rate of [Ca²⁺]i decline d[Ca²⁺]/dt and [Ca²⁺]i was obtained (Fig. 5 D), and data points from this relation were fitted to the following equation:

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d[Ca^{2+}]/dt = A[Ca^{2+}]^4 - L
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where A is a factor reflecting the rate of SR Ca²⁺ pumping, L represents the SR Ca²⁺ leak, and N is a power function related to the Ca²⁺ binding per SR pump unit (Klein et al., 1991). N = 4.03 ± 0.24 (2 to 39 curve fits) when allowed to fit freely, which is similar to what others have reported (Klein et al., 1991; Westerblad and Allen, 1993; Head et al., 2015). To facilitate the comparison of L and A between groups, N was set to four for all fibers, and values for L and A in each fiber were obtained when d[Ca²⁺]/dt − A[Ca²⁺]⁴ + L = 0. This assessment gave about twice as large SR Ca²⁺ leak (L) in 3-wk runners than in sedentary controls, whereas the leak in 6-wk runners was similar to controls (Fig. 5 E).

Figure 6. Pharmacologically destabilizing RYR1 causes increased baseline [Ca²⁺], with no negative effects on tetanic [Ca²⁺] or contractile force. (A) Original blots (top) and boxplot data (below) of immunoprecipitated RYR1 blotted for coprecipitated FKBP12 in tibialis anterior muscles from sedentary mice injected with NaCl, rapamycin (Rap), or SLF every 48 h for 3 wk (n = 11, 5, and 6, respectively). (B) Boxplots of baseline [Ca²⁺] in dissected single FDB fibers from NaCl- and rapamycin-injected mice (n = 7 vs. 5, from four mice). (C) Dissected single fiber from mice injected for 3 wk with rapamycin (light red circles) show no difference in tetanic force or tetanic [Ca²⁺] compared with NaCl-injected controls (open circles). Mean (± SEM) n = 7 vs. 5, from four mice. * P < 0.05 Rap or SLF vs. controls (NaCl) with one-way ANOVA. Box plots show median, quartiles, and min/max.
Calcium leak drives mitochondrial biogenesis

To investigate a causative role of increases in SR Ca\textsuperscript{2+} leak and mitochondrial biogenesis, resulting in increased muscle fatigue resistance and improved performance during a treadmill running exercise test. Moderation of FKBP12 from RYR1 resulting in a mild form of SR Ca\textsuperscript{2+} leak accompanied by cell signaling that promotes mitochondrial biogenesis, resulting in increased muscle fatigue resistance and improved performance during a treadmill running endurance exercise test. Modifications of RYR1 resulting in leaky SR seems to be a common feature following endurance exercise, where the severity of RYR1 modification relates to the intensity of the exercise performed.
Interestingly, RYR1 fragmentation was not observed with the low-intensity exercise used in the present study. Moreover, RYR1 fragmentation did not occur in vastus lateralis muscles of recreationally active subjects running a marathon, where instead FKBP12 dissociation from RYR1 was observed (Place et al., 2015). A likely scenario underlying the difference is that the HIIT-induced RYR1 fragmentation is linked to a very rapid production of reactive oxygen/nitrogen species and the rate of production of these highly reactive molecules is much lower during exercises at lower intensities (Sakellarious et al., 2013). Interestingly, RYR1 fragmentation was not observed after a HIIT session in vastus lateralis muscles of elite endurance athletes (Place et al., 2015), and we here show that FKBP12 association to RYR1 returned to preexercise levels and baseline [Ca$^{2+}$], was lowered in muscles of 6-wk running mice despite continued running at almost constant distance (see Fig. 1). Thus, RYR1 is generally less susceptible to modifications in the endurance-trained state, both regarding fragmentation after HIIT exercise and FKBP12 dissociation from RYR1 with low-intensity exercise.

In the short term, baseline [Ca$^{2+}$], in skeletal muscle fibers reflects the balance between Ca$^{2+}$ fluxes in and out of the SR, whereas in the longer term Ca$^{2+}$ fluxes over the sarcolemma become more important (Rios, 2010). There were no major differences between muscle fibers of control mice and 3- and 6-wk runners in the total SR Ca$^{2+}$ content as judged from similar [Ca$^{2+}$], during tetanic contractions at 150 Hz in the presence of caffeine (see Fig. 4 A), during which most Ca$^{2+}$ in the SR is released to the cytosol (Allen and Westerblad, 1995). In muscles of 3-wk runners, we observed FKBP12 dissociation from RYR1 accompanied by SR Ca$^{2+}$ leak, promoting an increase resting [Ca$^{2+}$]. At the same instance, SERCA1 was up-regulated and STIM1 down-regulated and both these protein changes act toward decreased resting [Ca$^{2+}$]. The net effect of all these changes was a slight (∼25%) but significant increase in resting [Ca$^{2+}$], in FDB fibers of 3-wk runners (see Fig. 1 C). Conversely, in muscles of 6-wk runners, FKBP12’s association with RYR1 had returned to pretraining levels, the protein expression of SERCA1 and STIM1 was further increased and decreased, respectively, and measured SR Ca$^{2+}$ uptake rate was higher than sedentary controls. Accordingly, resting [Ca$^{2+}$], was lower than the pretraining level in FDB fibers of 6-wk runners. Furthermore, the increased Ca$^{2+}$ entry seen after repeated stimulation became less pronounced with endurance exercise (see Fig. 3 B). This complex adaptive pattern underscores the fundamental importance of tightly controlled cellular Ca$^{2+}$ handling. Thus, a modest increase in resting [Ca$^{2+}$], emerges as a key trigger of beneficial adaptations such as more tightly controlled Ca$^{2+}$ homeostasis and increased fatigue resistance, whereas large and prolonged increases are associated with deleterious changes and muscle pathologies (Aydin et al., 2008; Bellinger et al., 2008, 2009; Andersson et al., 2011; Lanner, 2012; Waning et al., 2015; Matecki et al., 2016). In line with this, increased SOCE has been shown to promote skeletal muscle growth and endurance (Wei-LaPierre et al., 2013), but it is also linked to various myopathies (Pan et al., 2014).

Increased baseline [Ca$^{2+}$], can increase mitochondrial biogenesis in vitro (Ojuka et al., 2002, 2003; Wright et al., 2007). Furthermore, Lee et al. (2014) showed that pharmacologically destabilizing RYR1 can induce protein synthesis in skeletal muscle and boost endurance training adaptation in mice. A link between mild SR Ca$^{2+}$ leak and improved muscle function has also been observed in mice exposed to a cold environment, where FDB muscles showed increased oxidative capacity and improved endurance (Aydin et al., 2008; Brutton et al., 2010). Similarly, mutations in the ATCN3 gene resulting in α-actinin-3 protein deficiency, prevalent among elite endurance athletes, are coupled to increased SR Ca$^{2+}$ leak (Yang et al., 2003; Head et al., 2015). However, there is fine balance between beneficial and deleterious effects of SR Ca$^{2+}$ leak. Deleterious effects are, for instance, observed in soleus muscle partaking in the thermogenic response in cold-exposed mice, which display severe FKBP12 dissociation from RYR1 and markedly reduced force production; i.e., a hallmark of muscle “overtraining” (Aydin et al., 2008).

[Ca$^{2+}$]i is generally regarded as a broad second messenger, being initiated by a host of different receptors and which functions as an activator for several different downstream signaling cascades (Clapham, 2007). There are a number of potential pathways through which Ca$^{2+}$ could affect transcription of mRNAs related to oxidative capacity, including signaling dependent on Ca$^{2+}$-calmodulin kinase II, calcineurin, and cAMP response element–binding protein (Wu et al., 2002; Tavi and Westerblad, 2011; Ito et al., 2013; Bruno et al., 2014). These are relatively slow Ca$^{2+}$ sensors and are unlikely to respond to the transient increase in [Ca$^{2+}$], during individual contractions (Tavi and Westerblad, 2011). However, these Ca$^{2+}$ sensors might be activated by the repeated contractions produced during endurance exercise. Intriguingly, we here show similar increases in markers of mitochondrial biogenesis and fatigue resistance in muscles of mice performing wheel running and in sedentary mice exposed to the RYR1 destabilizing drugs rapamycin and SLF, which suggests a greater importance of a prolonged but modest increase in resting [Ca$^{2+}$], than the transient increases in [Ca$^{2+}$], during the repeated contractions of running. Moreover, wheel running requires a large increase in energy consumption, whereas the increase in energy metabolism with RYR1-destabilizing drugs would be small, although some increase would be expected due to increased SR Ca$^{2+}$ pumping to balance the increased SR Ca$^{2+}$ leak. Thus, these data indicate that the Ca$^{2+}$-related signaling was more important for the observed increases mitochondrial biogenesis and fatigue resistance than signaling induced by energy metabolic stress (e.g., activation of AMP-kinase; Kahn et al., 2005). Accordingly, the running distance remained constant after 3 wk and at 6 wk of training, resting [Ca$^{2+}$], was lower than the preexercise level, and the energy requirement would be expected to be similar to that at 3 wk. At this point, the muscles had apparently entered a new stable state with increased fatigue resistance, but without gene activation toward further adaptations (see Fig. 1). This fits with a general pattern where the accumulating effect of running-induced bursts of, for instance, mRNA for PGC1α leads to an increase in PGC1α protein, which after some delay results in an increased concentration of mitochondrial proteins. When the running exercise proceeds at a constant level, the amplitude of the mRNA bursts gradually declines while the content of mitochondrial proteins remains elevated (Perry et al., 2010). Moreover, the cellular mitochondrial content...
depends on the balance between the rates of synthesis (mitochondrial biogenesis) and removal of dysfunctional or damaged mitochondria via a selective degradation process known as mitophagy (Hood et al., 2018). In untrained muscle, acute endurance exercise has been shown to increase the rate of mitophagy (Vainshtein et al., 2015; Chen et al., 2018). In endurance-trained muscles, on the other hand, recent studies indicate a reduced basal rate of mitophagy as well as a blunted mitophagic response to acute endurance exercise, which possibly reflects an improved health of the mitochondrial pool in trained muscle (Schwalm et al., 2017; Carter et al., 2018; Chen et al., 2018; Kim et al., 2018). Thus, a decreased rate of mitochondrial degradation via mitophagy might explain how an increased cellular mitochondrial content can be maintained during constant running exercise despite signaling promoting mitochondrial biogenesis being returned to the untrained level.

Intriguingly, mice exposed to the RYR1-destabilizing drugs rapamycin and SLF, as well as mice having access to a running wheel, performed better than controls in the treadmill running exhaustion test (see Fig. 8 B), which is considered to provide an indirect measure of mouse VO2max (Kemi et al., 2002). The ability of the heart to deliver O2 to the working muscles is generally believed to be the limiting factor for VO2max (Levine, 2008). Running wheel exercise is likely to increase cardiac pumping capacity, but it is unclear as to how exposure to RYR1-destabilizing drugs without concomitant endurance exercise would improve cardiac function. One possible mechanism would be that exposure to these drugs improve cardiomyocyte function via Ca2+-dependent signaling similar to that in skeletal muscle fibers, although an increased SR Ca2+ leak induced by dissociation of FKBP 12.6 from RYR2 in cardiomyocytes is generally associated with impaired cardiac function and heart disease (Lehnart et al., 2004; MacMillan, 2013). Alternatively, the improved performance in the treadmill running exhaustion test reflects the increased mitochondrial capacity and fatigue resistance in skeletal muscles. Mice exposed to the RYR1-destabilizing drugs rapamycin and SLF showed increased baseline [Ca2+]i, together with increases in mRNA expression of the mitochondrial biogenesis–promoting genes PGC1α and TFAM. This was followed by increased mitochondrial oxidative capacity, increased fatigue resistance, and improved performance during endurance exercises.

Conclusion
In conclusion, endurance exercise induces RYR1 modifications and a modest SR Ca2+ leak in skeletal muscle fibers. Leaky RYR1 and the subsequent increase in baseline [Ca2+]i, trigger expression of mitochondrial biogenesis–promoting genes, resulting in increased oxidative capacity, increased fatigue resistance, and improved performance during endurance exercises.

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