Muscle sarcoplasmic reticulum calcium regulation in humans during consecutive days of exercise and recovery


Department of Kinesiology, University of Waterloo, Waterloo, Ontario, Canada

Submitted 20 April 2007; accepted in final form 4 July 2007

Duhamel TA, Stewart RD, Tupling AR, Ouyang J, Green HJ. Muscle sarcoplasmic reticulum calcium regulation in humans during consecutive days of exercise and recovery. J Appl Physiol 103: 1212–1220, 2007. First published July 26, 2007; doi:10.1152/japplphysiol.00437.2007.—The study investigated the hypothesis that three consecutive days of prolonged cycle exercise would result in a sustained reduction in the Ca²⁺-cycling properties of the vastus lateralis in the absence of changes in the sarcoplasmic (endoplasmic) reticulum Ca²⁺-ATPase (SERCA) protein. Tissue samples were obtained at preexercise (Pre) and postexercise (Post) on day 1 (E1) and day 3 (E3) and during recovery day 1 (R1), day 2 (R2), and day 3 (R3) in 12 active but untrained volunteers (age 19.2 ± 0.27 yr; mean ± SE) and analyzed for changes (nmol·mg⁻¹·min⁻¹) in maximal Ca²⁺-ATPase activity (Vₘₐₓ), Ca²⁺ uptake and Ca²⁺ release (phase 1 and phase 2), and SERCA isoform expression (SERCA1a and SERCA2a). At E1, reductions (P < 0.05) from Pre to Post in Vₘₐₓ (150 ± 7 vs. 121 ± 7), Ca²⁺ uptake (7.79 ± 0.28 vs. 5.71 ± 0.33), and both phases of Ca²⁺ release (phase 1, 20.3 ± 1.3 vs. 15.2 ± 1.1; phase 2, 7.70 ± 0.60 vs. 4.99 ± 0.48) were found. In contrast to Vₘₐₓ, which recovered at Pre E3 and then remained stable at Post E3 and throughout recovery, Ca²⁺ uptake remained depressed (P < 0.05) at E3 Pre and Post and at R1 as did phase 2 of Ca²⁺ release. Exercise resulted in an increase (P < 0.05) in SERCA1a (14% at R2) but not SERCA2a. It is concluded that rapidly adapting mechanisms protect Vₘₐₓ following the onset of regular exercise but not Ca²⁺ uptake and Ca²⁺ release.

short-term training; calcium-adenosinetriphosphatase; calcium uptake; calcium release; SERCA isoforms

IT IS CLEAR that at least in humans, a session of prolonged submaximal exercise results in alterations in sarcoplasmic reticulum (SR) Ca²⁺-cycling properties. The alterations in SR Ca²⁺-cycling properties consist of reductions in maximal Ca²⁺-ATPase sarcoplasmic (endoplasmic) reticulum (SERCA) activity (Vₘₐₓ) and reductions in Ca²⁺ uptake and Ca²⁺ release (3, 8, 10, 11, 34, 57). These alterations appear to occur in the absence of changes in the Ca²⁺ sensitivity of the Ca²⁺-ATPase (Ca₃⁺ pump) as measured by the free Ca²⁺ concentration ([Ca²⁺]j) necessary to elicit 50% Vₘₐₓ (Cas0) and the Hill coefficient (nH), defined as the slope of the relationship between [Ca²⁺]j and Ca²⁺-ATPase activity (8–11). Moreover, under normal dietary patterns, the preponderance of evidence indicates that the efficiency of SR Ca²⁺ transport as measured by the ratio of Ca²⁺ uptake to Ca²⁺-ATPase activity, commonly alluded to as the coupling ratio, is also unchanged (8, 9, 11).

Unclear are the effects of repetitive days of prolonged exercise on SR Ca²⁺-cycling responses. Two primary different responses could occur. On the one hand, the disturbances in SR Ca²⁺-cycling behavior could persist and, in fact, become exacerbated with additional exercise. Conversely, adaptive processes could be initiated, resulting in changes in protein abundance, potentially offsetting the exercise-induced disturbances in SR Ca²⁺-cycling properties. Evidence exists for both possibilities.

The acute effects of exercise have been shown to result in structural alterations to both the Ca²⁺ pump and the calcium release channel, commonly labeled the ryanodine receptor (RyR). In the case of the Ca²⁺ pump, both prolonged exercise (36) and chronic low-frequency electrical stimulation (CLFS) (14, 38, 40) result in changes in the region of the adenine nucleotide site, ostensibly due to oxidation and nitrosylation secondary to damage from the accumulation of reactive oxygen species (ROS) (32, 39). The accumulation of ROS during contractile activity also remains as an inviting theory to explain the structural abnormalities that occur to the RyR (16).

Recovery of Ca²⁺-ATPase activity appears to take considerable time after exercise. In muscles of both animals subjected to CLFS (38) and voluntary exercise in humans (57), recovery of Vₘₐₓ was not complete for at least 36 h following exercise. In the absence of adaptive changes, a failure of Vₘₐₓ to fully recover could lead to greater compromises in SR Ca²⁺-cycling during subsequent exercise.

Prolonged contractile activity is also known to act as a powerful stimulus for remodeling of the skeletal muscle cell, including the properties of the SR. Of the few nonhuman studies that have addressed training-induced effects on the SR in mammalian muscle, it has been found that reductions in Ca²⁺-ATPase protein (21), Ca²⁺ uptake (30, 31), Vₘₐₓ (30), and Ca²⁺ release (30) occur. Interestingly, at least for the training protocols employed for the rat, it appears that the adaptations are more pronounced in muscle composed of a large proportion of fast-twitch fibers (30). In the case of Ca²⁺-ATPase, measurements of mRNA also support that the changes are specific to fast-twitch-based muscles and to the SERCA2a isoform (33).

Adaptations in the SR with training also appear to extend to humans. In the only longitudinal study published to date, we (19) have reported that 10 wk of prolonged submaximal exercise results in reductions in Vₘₐₓ, Ca²⁺ uptake, Ca²⁺ release, and SERCA1a in vastus lateralis. No changes occurred in SERCA2a. The adaptation in the SERCA isoforms appears to begin early. Sixteen sessions of heavy intermittent exercise performed over 16 h resulted in a small but significant increase in SERCA2a and a decrease in SERCA1a (57). Expectations of large increases in SERCA expression during the first few days...
of exercise may be unrealistic given the relatively slow turn-over rate of the protein (37).

The purpose of this study was to investigate the effects of repeated days of prolonged exercise and recovery on SR Ca\(^{2+}\)-cycling properties in skeletal muscle. We have hypothesized that the acute reductions in Ca\(^{2+}\)-ATPase activity, Ca\(^{2+}\) uptake, and Ca\(^{2+}\) release observed during the first day of exercise would persist throughout the third consecutive day of exercise, resulting in greater disturbances in these properties following exercise. By the third day of inactivity, all SR properties would recover to preexperimental levels. The changes in SR Ca\(^{2+}\)-ATPase will not be accompanied by changes in SERCA1a or SERCA2a protein abundance.

METHODS

Participants

The volunteers consisted of 12 active students with a mean age and body mass of 19.2 ± 0.27 yr and 71.1 ± 3.4 kg, respectively. Peak aerobic power (\(V_{\text{O2peak}}\)) measured during a progressive cycle protocol to fatigue was 44.8 ± 2.0 ml·kg\(^{-1}\)·min\(^{-1}\). All volunteers were healthy and free of any medication as determined by questionnaire. Although participants were recreationally active, they did not participate in prolonged exercise involving large muscle group action on a regular basis. The overall description of the study, the specific protocols to be employed, and the associated risks were described during a familiarization session before obtaining written consent. The study was approved by the Office of Research Ethics at the University of Waterloo before written consent was obtained from the volunteers.

Experimental Design

Each participant was required to visit the laboratory on at least seven different occasions to complete all testing and measurements. On the first visit, which occurred ~2 wk before the start of the experiment, \(V_{\text{O2peak}}\) was measured. The next six visits were used to examine the SR Ca\(^{2+}\)-cycling responses to consecutive days of exercise and recovery. Prolonged submaximal exercise was performed on day 1 (E1), day 2 (E2), and day 3 (E3) in the morning at approximately the same time. The exercise task consisted of cycling at ~60% \(V_{\text{O2peak}}\) for a maximum of 2 h. For subjects who were not able to complete 2 h at E1, the same time was used for exercise at E2 and E3. Recovery characteristics were examined ~24 h following E3 (R1) and at similar times on day 2 (R2) and day 3 (R3). No exercise was performed on the recovery days. Participants were also requested to refrain from exercise for at least 48 h before E1.

No dietary manipulation was applied during the days leading into the initial training session or during the experimental period. During these periods, the participants were requested to maintain their normal dietary habits. However, ~2 h before reporting to the laboratory on the exercise days, participants were provided with and instructed to ingest a can of Ensure (250 kcal) meal replacement (Ross Products Division, Saint-Laurent, PQ, Canada). This practice was followed to standardize the nutrient intake before the prolonged exercise that occurred in the morning. No other nutrients (solid foods or beverage) was allowed before or during the testing. All participants were requested to abstain from caffeine and alcohol for at least 24 h before exercise at E1 and throughout the 6-day experimental period. On the experimental days, both exercise and recovery, temperatures ranged between 23 and 24°C and relative humidity between 50 and 60%. We have selected the three-consecutive-day model of prolonged exercise on the basis of a number of previous studies that have demonstrated rapid adaptations in muscle metabolic behavior (18, 22) and Na\(^{+}\)-K\(^{+}\)-ATPase concentration (20) within the first few days of regular exercise. The 3 days of inactivity selected for study was designed to allow for recovery of the changes in SR Ca\(^{2+}\)-cycling properties induced by the last session of exercise and the expression of SERCA protein isoforms.

Exercise Protocols

The progressive exercise test for measurement of \(V_{\text{O2peak}}\) was as previously employed in our laboratory (27) and included a 4-min period of cycling at 25 W followed by 15-W step increases in power output (PO) each minute until fatigue. Gas exchange was monitored throughout the protocol (28) and used to both characterize the relationship between PO and \(O_2\) consumption (\(V_{\text{O2}}\)) and to assess \(V_{\text{O2peak}}\). The PO-\(V_{\text{O2}}\) relationship was used to establish the PO designed to elicit ~60% \(V_{\text{O2peak}}\) in each volunteer. All exercise was performed upright on an electrically braked cycle ergometer (Quinton 870) that was calibrated on a daily basis. The seat height was standardized for each participant for both the progressive and prolonged exercise protocols.

The prolonged exercise protocol was designed to allow for the investigation of exercise-induced changes in the SR Ca\(^{2+}\)-cycling properties and the response of these properties during the recovery days. Before beginning the exercise, preparations were made for tissue sampling from the vastus lateralis muscle. For tissue sampling, seven separate sites were prepared, randomized between legs. Four sites were used for tissue sampling during the exercise days (Pre, Post on E1 and E3) and three sites for sampling during the recovery days, one per day. The location and preparation of the sites and the technique used for tissue sampling were as previously employed by our group (18, 22). In brief, on a given experimental day, the sites were selected (2 in the case of the exercise days, 1 per leg), and incisions were made after local anesthesia (2% xylocaine with epi-nephrine) was applied. Two separate samples were extracted from each site using suction to increase tissue yield. The tissue samples were obtained within 5 min of beginning exercise (Pre) and immediately following the exercise (Post).

Analytical Techniques

SR Ca\(^{2+}\)-ATPase properties. Whole muscle homogenates prepared from fresh tissue following extraction from the vastus lateralis and stored (−80°C) were used to determine the catalytic properties of the SR Ca\(^{2+}\)-ATPase. Homogenates were prepared using ~30 mg tissue diluted 1:11 (wt/vol) in ice-cold buffer containing (in mM) 250 sucrose, 5 HEPES, 10 Na\(_3\)S, and 0.2 phenylmethylsulfonyl fluoride (PMSF) (pH 7.5) by using a handheld glass homogenizer (Duell 20, Kontes). The catalytic activity was measured in duplicate at 37°C by using a spectrophotometric method originally developed by Simonides and van Hardeveld (51) with modifications (8, 45). The assay mixture (pH 7.0) contained (in mM) 200 KCl, 20 HEPES, 15 MgCl\(_2\), 1 EDTA, 10 Na\(_3\)S, 5 ATP, 0.3 NADH, 10 phosphoenolpyruvate (PEP), 18 U/ml lactate dehydrogenase (LDH), 18 U/ml pyruvate kinase (PK), and 25 μl homogenate. Ca\(^{2+}\)-dependent ATPase activity was measured by using 0.5-μl additions of 100 mM CaCl\(_2\). The additions were continued until a plateau and subsequent decline in Ca\(^{2+}\)-ATPase activity were observed. Ca\(^{2+}\)-independent or basal ATPase activity was determined by using 40 μM of the Ca\(^{2+}\)-ATPase inhibitor cyclopiazonic acid (CPA) (49). The Ca\(^{2+}\)-ATPase activity was calculated as the difference between the total ATPase activity measured without CPA and the basal ATPase measured with CPA. Ca\(^{2+}\)-ATPase activity was measured with and without 1 μM of the Ca\(^{2+}\) ionophore A-23187 (Sigma C-7522). The Ca\(^{2+}\) ionophore was added to prevent intraluminal Ca\(^{2+}\) accumulation in SR, which is inhibitory to Ca\(^{2+}\)-ATPase activity (52). By calculating the ratio of Ca\(^{2+}\)-ATPase activity, measured with and without the ionophore A-23187, it is possible to gain an indirect measure of the effects of exercise on changes in SR membrane integrity.

Measurement of [Ca\(^{2+}\)]\(_i\), used to determine the Ca\(^{2+}\) dependency of the Ca\(^{2+}\)-ATPase reaction, was performed with dual-wavelength spectrofluorometry and the Ca\(^{2+}\)-fluorescent dye indo-1. The excita-
tion wavelength was 355 nm, and the emission was measured at 405 and 485 nm for Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free forms of the dye, respectively. The dissociation constant used for the interaction between Ca\(^{2+}\) and indo-1 was 250 nM. Further details of the assay appear in earlier publications from our laboratory (55).

The kinetic properties measured included maximal Ca\(^{2+}\)-ATPase activity (\(V_{\text{max}}\)), the [Ca\(^{2+}\)]\(_r\) needed to produce 50% \(V_{\text{max}}\) (Ca\(_{50}\)), and the slope of the relationship between Ca\(^{2+}\)-ATPase activity and [Ca\(^{2+}\)]\(_r\) (Hill coefficient, \(n_H\)). \(V_{\text{max}}\) was defined as the peak Ca\(^{2+}\)-ATPase activity; Ca\(_{50}\) represented the [Ca\(^{2+}\)]\(_r\) obtained from a sigmoid fit of the data that yields 50% \(V_{\text{max}}\); and \(n_H\) was obtained through nonlinear regression, using a portion of the curve that corresponded to between 10 and 90% \(V_{\text{max}}\).

To assess whether phosphatase activation may occur during tissue preparation, potentially biasing out measurement of \(V_{\text{max}}\), we have used a phosphatase inhibitor cocktail (Sigma P2850) added during the homogenization procedure using tissue obtained from a related experiment on humans. A comparison of \(V_{\text{max}}\) with and without the phosphatase inhibitor indicated no systematic difference between conditions either at rest or during exercise (differences ranging between 2.0% and 6.3%) (H. J. Green, unpublished observation).

**SR Ca\(^{2+}\) uptake and Ca\(^{2+}\) release.** Ca\(^{2+}\) uptake and Ca\(^{2+}\) release were determined in a common assay using homogenates prepared from fresh tissue that was frozen and stored in conjunction with the homogenates used for Ca\(^{2+}\)-ATPase determinations. Ca\(^{2+}\) uptake and Ca\(^{2+}\)-release rates were determined in duplicate at 37°C. The reaction buffer consisted of (in mM) 200 KCl, 20 HEPES, 15 MgCl\(_2\), 5 oxalate, 10 PEP, 18 U/ml LDH, 18 U/ml PK, and 0.0015 indo-1. To produce a constant starting [Ca\(^{2+}\)]\(_r\) of \(\sim 3.5 \mu M\), 2.5 \(\mu M\) of CaCl\(_2\) (10 mM) was added to the cuvette containing 2 ml of the reaction buffer. Ca\(^{2+}\) uptake was initiated by adding 5 mM ATP. The rate of Ca\(^{2+}\) uptake was determined at 2,000 nM by differentiating a linear fit curve with [Ca\(^{2+}\)]\(_r\) measured using indo-1, as earlier described.

After Ca\(^{2+}\) uptake had stabilized, Ca\(^{2+}\) release was initiated by adding 20 \(\mu M\) of 4-chloro-m-cresol (4-CMC), a highly specific Ca\(^{2+}\)-releasing agent (23). As described previously (56), we have measured two phases of Ca\(^{2+}\) release, namely phase 1 and phase 2. With our procedures, we consistently find that 4-CMC induces a biphasic Ca\(^{2+}\) release, which can be detected by distinct differences in slope. The rapid early phase (phase 1) produces the most marked slope, while the more delayed later phase (phase 2) has a lower slope. Maximal Ca\(^{2+}\)-release rates for each phase were calculated using the same method as for Ca\(^{2+}\) uptake and differentiating a linear fit curve.

The ratio between Ca\(^{2+}\) uptake (2,000 nM) and \(V_{\text{max}}\) was determined and labeled the apparent coupling ratio (12). We have called this the apparent coupling ratio because of the different assay conditions used to determine each property. For Ca\(^{2+}\) uptake, a precipitating agent (oxalate) had to be employed to bind Ca\(^{2+}\) inside the SR to prevent back-inhibition (41). For Ca\(^{2+}\)-ATPase activity, a membrane permeabilizing agent was necessary to also prevent back-inhibition (52). We also emphasize that given the limited sensitivity of indo-1, it is not clear if 2,000 nM was sufficient to produce a maximal Ca\(^{2+}\) uptake rate.

For all SR properties, protein was determined in duplicate by the method of Lowry as modified by Schacterle and Pollock (47). Ca\(^{2+}\)-ATPase isoforms. Detection of Ca\(^{2+}\)-ATPase isoform distribution (SERCA1a and SERCA2a) was performed using electrophoresis and Western blotting techniques, consistent with procedures previously detailed in our laboratory (19). In brief, 10 \(\mu g\) protein, obtained from a postnuclear homogenate, was electrophoresed with 7.5% SDS-polyacrylamide gels (Bio-Rad Mini-PROTEAN II), equilibrated (15 min) in cold transfer buffer (25 mM Tris, 192 mM glycine, and 20% vol/vol methanol), and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) by placing the gels in transfer buffer and applying high voltage (23 V) for 40 min (Trans-Blot Cell, Bio-Rad). The postnuclear homogenate was obtained from the supernatant of frozen tissue, which had been initially prepared in a buffer consisting of 5 mM HEPES (pH 7.5), 250 mM sucrose, 0.2% NaN\(_3\), and 0.1 \(\mu M\) PMSF in a ratio of 15:1 (vol/wt) and then extracted by adding an equal volume of buffer containing 10 mM sodium phosphate (pH 7.4), 150 mM NaCl, 2% Triton X-100, 2% deoxycholate, 0.2% SDS, 0.2 mM PMSF, and 1,000 IU aprotinin. To obtain the postnuclear homogenate, this mixture was centrifuged at 10,000 g for 15 min, and the supernatant was withdrawn and stored at \(-80°C\) pending analysis. It has been reported that 95–99% of the SERCA protein remains in the supernatant after this procedure (58).

The PVDF membranes were blocked for nonspecific binding by using a 10% skim milk powder in Tris-buffered saline (pH 7.5) applied overnight at room temperature, before a 60-min incubation at room temperature with the primary monoclonal antibodies for SERCA1a (A52) and SERCA2a (7E6) obtained from Affinity Bioreagents using dilution of 1:2,500 (SERCA1a) and 1:1,000 (SERCA2a).

After washing with Tris-buffered saline and 0.1% Tween 20 (TBS-T), the PVDF membranes were incubated for 60 min at room temperature with a secondary antibody (anti-mouse IgG, conjugated to horseradish peroxidase) and protein determined by densitometry and an enhanced chemiluminescence immunodetection procedure (Amer sham ECL-RPN2106 P1), and visualized using the Chemi Genius imaging system and Gene Snap software (SynGene). Densitometry measurements were performed using Gene Tools software (Syngene). Signal intensity of the blots were found to be linear over the protein range employed in our experimental conditions.

All samples obtained at rest (before exercise in the case of E1 and E3) for a given subject were run in duplicate on separate gels along with the standard on the same day. The average value of the duplicate measurements were initially expressed as a percentage of the standard and then as a percentage of the initial control value obtained before exercise on E1. A sample of tissue obtained from human vastus lateralis was used as the standard. Multiple aliquots obtained from the postnuclear homogenate were stored at \(-80°C\) and used as a standard during each analytical session.

**Statistics**

The data were analyzed using one-way ANOVA procedures for repeated measures. The one-way ANOVA was used to investigate the effects of the exercise (Pre, Post) at E1 and E3 and the effect of the recovery days (R1, R2, R3). Where significant differences were found, the Neuman-Keuls technique was applied to determine which means were different. Significance was set at the 0.05 level. Throughout the text, data are expressed as means \(\pm\) SE. Where a difference between means is indicated in the text, significance is implied.

**RESULTS**

**SR Changes**

Exercise at E1 but not E3 resulted in a reduction in \(V_{\text{max}}\). The reduction in \(V_{\text{max}}\) occurred in the absence of changes in Ca\(^{2+}\) sensitivity as measured by both Ca\(_{50}\) and \(n_H\) (Fig. 1). Before the third day of exercise, \(V_{\text{max}}\) had recovered to preexperimental levels. No differences were observed in \(V_{\text{max}}\) between R1, R2, and R3 or between the recovery days and E1 and E3 at Pre. Exercise, either on E1 and E3, did not alter the ionophore ratio. Similarly, the ionophore ratios were not different before exercise on the exercise days or between any of the recovery days (Table 1).

As with \(V_{\text{max}}\), exercise reduced Ca\(^{2+}\) uptake by 26.7% on E1 (Fig. 2). Unlike \(V_{\text{max}}\), however, Ca\(^{2+}\) uptake remained depressed by 20% before exercise on E3. At E3, exercise failed to elicit further reductions in Ca\(^{2+}\) uptake. Following E3, Ca\(^{2+}\) uptake was not different from the control value, and there were no differences on the recovery days.

**REFERENCES**


uptake remained depressed until R2. At R2 and R3, Ca\textsuperscript{2+} uptake was not different compared with preexercise on E1. At postexercise on E1 and E3, Ca\textsuperscript{2+} uptake was lower than at R2 and R3.

The apparent coupling ratio, defined as the ratio between Ca\textsuperscript{2+} uptake and $V_{\text{max}}$, was unchanged either with exercise on E1 and E3 or at rest between any of the exercise and recovery days (Table 1). However, lower apparent coupling ratios were observed at Post R3 compared with Pre E1 and between Post E3 and R3.

Ca\textsuperscript{2+} release phase 1 and phase 2 were reduced by 25.1% and 35.2%, respectively, during exercise on E1 (Fig. 3). Exercise at E3 failed to elicit changes in either phase 1 or phase 2 Ca\textsuperscript{2+} release. However, unlike phase 1, which showed no difference between preexercise levels at E1 and E3, phase 2 Ca\textsuperscript{2+} release before exercise on E3 remained depressed. Although there was a trend for phase 2 Ca\textsuperscript{2+} release to remain depressed throughout the recovery days compared with E1, the difference was not significant.

The consecutive days of exercise also resulted in an approximate 14% increase in SERCA1a (Fig. 4). However, the increase was not observed until 2 days following the last exercise session. This increase was rapidly reversible with values at R3 not different from E1. No changes in SERCA2a were observed between the exercise and recovery days.

**DISCUSSION**

This study has both confirmed previous observations regarding the reduction in SR Ca\textsuperscript{2+}-cycling properties during the initial session of exercise and, in addition, generated several novel findings regarding the response of SR Ca\textsuperscript{2+}-cycling properties to repeated days of exercise and recovery in human skeletal muscle. Unexpected and inconsistent with our hypothesis was the effect of exercise on the SR Ca\textsuperscript{2+}-cycling properties on the third consecutive day. We have found that neither $V_{\text{max}}$, Ca\textsuperscript{2+} uptake, nor Ca\textsuperscript{2+} release, both phase 1 and phase 2, were reduced by the exercise. Given that the intensity and duration were comparable across the 3 days of exercise, it would appear that adaptations have resulted to protect these SR Ca\textsuperscript{2+}-cycling properties from the deleterious effects observed during the initial insult. However, in the case of Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+} release (phase 2), the protection comes at the expense of a lower preexercise value.

The recovery characteristics of the different Ca\textsuperscript{2+}-cycling properties during and following the three consecutive days of exercise also demonstrate novel insights. As postulated, all three properties recovered by the third day of inactivity. However, differences were observed between the properties in the time course of recovery between the consecutive days of exercise and the consecutive days of inactivity. Depressions in Ca\textsuperscript{2+} uptake persisted during the first 2 days of exercise and during the first day of recovery, while the depression in Ca\textsuperscript{2+} release (phase 2) was only evident during days of exercise. A strong trend was observed for phase 1 to be depressed during the period as well, but the differences were not significant. $V_{\text{max}}$ was not different at rest between any of the exercise or recovery days. Collectively, these results indicate a dissociation between the recovery kinetics of $V_{\text{max}}$ and Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+} release following an exercise protocol. It should be emphasized that the changes in $V_{\text{max}}$ and Ca\textsuperscript{2+} uptake either with exercise or recovery were not accompanied by changes in membrane permeability as measured by the ionophore ratio. A cumulative effect of the repetitive days of exercise appears to change the efficiency of Ca\textsuperscript{2+} sequestration as measured by the
apparent coupling ratio since the value following exercise on day 3 was lower than the value observed before exercise on day 1.

Our results indicate that in response to three consecutive days of prolonged submaximal exercise, an increase in the fast isoform of the Ca\(^{2+}\)/H\(^{+}\)-ATPase, namely SERCA1a but not SERCA2a, occurs. Moreover, the increase is transient, not evident until 2 days following the last exercise session before reversing by 3 days of recovery. To our knowledge this is only the third study investigating isoform adaptability in SR Ca\(^{2+}\)-cycling proteins in human skeletal muscle in response to regular exercise. In a previous study from our laboratory, we (19) reported a decrease in the SERCA1a isoform and no change in the SERCA2a isoform in response to a progressive 10-wk program of prolonged heavy cycle exercise. In the additional study, a 5-wk program of sprint training resulted in an increase in the relative density of the SERCA1a isoform (44). These two studies suggest that at least over a more extended training period, the Ca\(^{2+}\)-ATPase may adapt according to the demands placed on SR Ca\(^{2+}\) cycling. High-intensity exercise resulting in a demand for rapid Ca\(^{2+}\) cycling, both Ca\(^{2+}\) release and Ca\(^{2+}\) uptake, promotes increases in both the relative densities of RyR and the SERCA1a isoform (44). These adaptations promote a phenotype approaching that observed in type II or fast-twitch fibers, which possess much higher densities of these proteins, consistent with the need for a rapid regulation of the Ca\(^{2+}\) transient given the rapid velocities that these fibers are capable of generating (4). However, it must be emphasized that since the vastus lateralis is composed of ~50% of type I and type II fibers (46), it is not clear if changes observed in SERCA1a and RyR following training was specific to one fiber type or altered the abundance in both fiber types. In this regard, it was reported that the sprint training also resulted in increases in SERCA2a (44), with the distribution by fiber type also unclear.

Table 1. Muscle sarcoplasmic reticulum coupling and ionophore ratios during consecutive days of exercise and recovery

<table>
<thead>
<tr>
<th></th>
<th>E1</th>
<th>E3</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionophore ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>5.33±0.30</td>
<td>4.58±0.36</td>
<td>4.75±0.34</td>
<td>5.01±0.23</td>
<td>5.00±0.21</td>
</tr>
<tr>
<td>Post</td>
<td>5.04±0.25</td>
<td>4.86±0.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apparent coupling ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>0.040±0.002</td>
<td>0.034±0.002</td>
<td>0.035±0.002</td>
<td>0.037±0.002</td>
<td>0.040±0.002†</td>
</tr>
<tr>
<td>Post</td>
<td>0.036±0.002</td>
<td>0.032±0.003*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 12). E1, E3, exercise on day 1 and day 3, respectively; R1, R2, R3, recovery on days 1, 2, and 3, respectively; Pre, preexercise; Post, postexercise. Apparent coupling ratio is defined as the ratio between Ca\(^{2+}\) uptake at 2,000 nM and maximal Ca\(^{2+}\)-ATPase activity (V\(_{\text{max}}\)). Ionophore ratio is defined as the ratio of V\(_{\text{max}}\) measured with the Ca\(^{2+}\) ionophore A-23157 and V\(_{\text{max}}\) measured without the ionophore. *Significantly different (P < 0.05) from Pre E1. †Significantly different (P < 0.05) from Post E3.
Given the demands imposed by our prolonged submaximal cycling protocol, we had expected that the isoform response would be in the direction of increased SERCA2a, typical of what has been reported in animals subjected to regular prolonged contractile activity. The unexpected increase that we have reported in SERCA1a with our exercise model could occur as a result of decreases in degradation, increases in synthesis, or a combination of both mechanisms. The increases in SERCA1a may be a response to the strain imposed early by the dramatic and sustained increase in contractile activity. Given the results of a previous study in which we (19) reported increases in SERCA2a with an extended training program, it might be expected that the SERCA isoform changes may display a time dependency, ultimately culminating in a shift toward the slow isoform.

It might be expected that increases in SERCA1a protein levels as we have observed in this study would result in an increase in both $V_{\text{max}}$ and Ca$^{2+}$ uptake, specifically at R2, the point at which the increase was detected. However, we found no change in either $V_{\text{max}}$ or Ca$^{2+}$ uptake at R2 compared with preexercise at E1. Furthermore, at R3, when SERCA1a regressed to preexercise levels, no differences existed between R2 and R3 for either $V_{\text{max}}$ or Ca$^{2+}$ uptake. The failure to find a coupled response of $V_{\text{max}}$ and Ca$^{2+}$ uptake with changes in SERCA1a could occur as a result of the insensitivity of our analytical measurements to detect change of the magnitude expected or because of incomplete recovery from the disturbing effects of exercise at E3. To examine the latter probability, we have incorporated a 3-day recovery period into our experimental design. Our results clearly demonstrate that $V_{\text{max}}$ recovered from the exercise-induced reduction observed at E1 and E3 within 24 h and then remained stable throughout the recovery days. Ca$^{2+}$ uptake, although displaying a slower recovery than $V_{\text{max}}$, was recovered to preexercise levels by R2 with no further change at R3. These results provide evidence that the dissociation that exists between the changes in SERCA1a and the Ca$^{2+}$-sequestering properties cannot be attributed to a failure to recover from the last exercise session.

In our previous training study (19), we found that the SR Ca$^{2+}$-sequestering properties, measured in tissue samples extracted ~4 days following the last training session, displayed an expected reduction in $V_{\text{max}}$ and Ca$^{2+}$ uptake consistent with the reduction in SERCA1a concentration. Two previous studies (30, 31) employing prolonged training in rats have also reported reductions in resting Ca$^{2+}$ uptake following training. However, the reduction was only observed in plantaris (30) and the superficial vastus lateralis (31) and not the soleus (30, 31) or the deep vastus lateralis (31), where no training changes were detected. Given the fiber-type composition of these muscles and regions (7), it would appear that the training effect was highly specific to muscles containing a predominance of type IIB or fast-glycolytic, low-oxidative based fibers. Interestingly, reductions in $V_{\text{max}}$ were observed with training in the plantaris but not the soleus muscle (30). Since acute exercise also resulted in reductions in $V_{\text{max}}$ and Ca$^{2+}$ uptake in both the plantaris and soleus muscles (30), it is not clear if the 48-h period provided from the last training session was sufficient to allow for full recovery of the enzyme. In one other study investigating training at SR properties in humans, it was found that resting $V_{\text{max}}$ and Ca$^{2+}$ uptake was depressed in endurance-trained subjects compared with untrained subjects (35). Since this was a cross-sectional study dealing with elite athletes, the differences could have existed before training.

In this study, we demonstrate a dissociation between $V_{\text{max}}$ and Ca$^{2+}$ uptake during recovery, with the recovery of Ca$^{2+}$ uptake being much more delayed. Several possibilities exist to explain this apparent contradiction. Conceivably, the dissociation could occur as a product of the different conditions used to measure each property. In the case of Ca$^{2+}$-ATPase activity, the Ca$^{2+}$-ionophore A-23187 was employed to prevent the accumulation of Ca$^{2+}$ in the lumen of the SR. The use of oxalate has been shown to increase Ca$^{2+}$-uptake rates in homogenates, supposedly by preventing the increases in SR luminal [Ca$^{2+}$]$_{i}$ (29). We also emphasize that while Ca$^{2+}$-ATPase was measured during maximal activating conditions, producing a clear $V_{\text{max}}$, Ca$^{2+}$ uptake was not. This is because of limitations in the sensitivity of the indo-1, the fluorescent dye used to measure [Ca$^{2+}$]$_{i}$. We routinely measure Ca$^{2+}$ uptake at Ca$^{2+}$ concentrations up to 2,000 nM, as we have in

---

**Fig. 4.** Representative Western blots (A) and relative changes in muscle sarcoplasm reticulum Ca$^{2+}$-ATPase (SERCA) concentration (B) during consecutive days of exercise and recovery. Values are means ± SE. Values are means ± SE in relative units. The values for E1 was set at 1.00; n = 12. Measurements at E1 and E3 were made before exercise. Western blot analyses of fast (SERCA1a; left) and slow (SERCA2a; right) Ca$^{2+}$-ATPase were made with the use of monoclonal antibodies A52 and 7E6. *Significantly different ($P < 0.05$) from E1. #Significantly different ($P < 0.05$) from R2.

---

**Fig. 4.** Representative Western blots (A) and relative changes in muscle sarcoplasm reticulum Ca$^{2+}$-ATPase (SERCA) concentration (B) during consecutive days of exercise and recovery. Values are means ± SE. Values are means ± SE in relative units. The values for E1 was set at 1.00; n = 12. Measurements at E1 and E3 were made before exercise. Western blot analyses of fast (SERCA1a; left) and slow (SERCA2a; right) Ca$^{2+}$-ATPase were made with the use of monoclonal antibodies A52 and 7E6. *Significantly different ($P < 0.05$) from E1. #Significantly different ($P < 0.05$) from R2.
this study, but it is not clear how close these measurements are to the maximal Ca\(^{2+}\) uptake.

We do not feel that these technical issues were significant, however, since the discrepancy between \(V_{\text{max}}\) and Ca\(^{2+}\) uptake only occurred under very specific conditions, namely during recovery. It is possible that the slower recovery observed for Ca\(^{2+}\) uptake following exercise could be explained by leakage of Ca\(^{2+}\) during uptake through the SR membrane or the RyR channel, resulting in a net reduction in Ca\(^{2+}\) uptake. Thermal stress, as an example, is known to increase leakage of Ca\(^{2+}\) from the lumen of the SR in the cytosol (6, 48, 50). In the absence of changes in \(V_{\text{max}}\), decreases in Ca\(^{2+}\) uptake should result in a reduction in the coupling ratio. This ratio, which we have defined as the apparent coupling ratio to reflect differences in the protocols used for measurement of \(V_{\text{max}}\) and Ca\(^{2+}\) uptake, was not observed to change, Pre to Post at E1 and E3 or at rest between the exercise and recovery days. However, a trend is clearly evident as indicated by the reduction in the apparent coupling ratio with exercise at E1 and E3. At Post E3, the apparent coupling ratio was lower than Pre E1, suggesting a cumulative effect of the consecutive days of exercise. In this regard, we would emphasize that in our many previous studies using prolonged exercise, we have not observed any change in the apparent coupling ratio when a normal diet is followed (8, 9, 19). However, exercise plus a 4-day period of a low-carbohydrate diet elicits modifications in the apparent coupling ratio (10, 11). In this study, since participants followed a normal carbohydrate diet, it would appear that the repeated sessions of exercise culminate in a lower apparent coupling ratio.

An inviting possibility to explain the apparent protection offered to \(V_{\text{max}}\) with consecutive days of exercise is via heat shock protein 70 (HSP70). This heat shock protein has been shown to bind to SERCA1a and prevent thermal activation (54). Given the intensity and prolonged nature of the exercise, substantial increases in muscle temperature would be expected (17). This would not seem a credible possibility, however, since the 12% increase observed in HSP at R1 was not significant (\(P = 0.11\)) (data not shown). Moreover, \(V_{\text{max}}\) recovered to resting levels before there was any indication of a change in HSP70.

Exercise at E1 resulted in pronounced reductions in both phase 1 and phase 2 Ca\(^{2+}\) release. Both phases of Ca\(^{2+}\) release appear to remain depressed before exercise at E3; however, only in the case of phase 2 was significance found. At E3, exercise failed to elicit further declines regardless of phase. Although a clear trend exists for Ca\(^{2+}\) uptake to remain depressed throughout the first 2 days of recovery and in the case of phase 1, throughout the 3-days of recovery, significance was not found. The depression in Ca\(^{2+}\) release during exercise was expected given the many previous studies that have reported a similar finding in humans (3, 8, 9, 12, 13, 25, 34, 35). The persistent reduction in Ca\(^{2+}\) release is a novel finding and suggests that recovery processes are not complete during the 24-h period between exercise sessions. Given that the reduction in Ca\(^{2+}\) release appears to be structural damage to the RyR (16), it would appear that repair of the damaged RyR or the synthesis of new RyRs is a more delayed process.

In previous training studies in humans, using a prolonged exercise protocol for 5 wk, a pronounced reduction in Ca\(^{2+}\) release was observed (19). A similar finding has been reported in a cross-sectional study employing trained endurance athletes and untrained controls (35). However, in rats subjected to a 10-wk endurance program, resting Ca\(^{2+}\) release was not altered either in the plantaris or soleus, two muscles composed of a predominance of fast- and slow-twitch fibers, respectively (30). The contradictory effect of training on Ca\(^{2+}\) release may be due to differences between species and/or the muscles examined.

In this study, unlike others (34), we have assessed Ca\(^{2+}\) release in two phases, namely phase 1 and phase 2, based on clear biphasic Ca\(^{2+}\)-release rate. We have examined the different phases in an earlier study using both 4-CMC and AgNO\(_3\), and found that with 4-CMC, which is highly specific to RyR, a sixfold higher release occurred (56). Moreover, we have found that ruthenium red, a known inhibitor of the RyR, reduced phase 1 by 59% and completely blocked phase 2 (56). The differential effects observed have led us to speculate that different mechanisms may be involved in the two phases of release. Consequently, and given that a clear demarcation exists between the two phases of Ca\(^{2+}\) release, we routinely report on both phases. Although most of our studies report a similar pattern of change for response to exercise for both phases, some differences have been reported. In this study, in general, the changes in phase 1 and phase 2 Ca\(^{2+}\) release are similar. However, there is a suggestion of a more delayed normalization of phase 2 Ca\(^{2+}\) release in recovery and particularly at E3. It is unclear what the physiological significance of this difference is, if any.

In summary, we have shown that, as expected, prolonged exercise in the unacclimatized results in a disturbance in SR Ca\(^{2+}\)-cycling function, as evidenced by the decrease in \(V_{\text{max}}\), Ca\(^{2+}\) uptake, and Ca\(^{2+}\) release. A similar session of exercise performed on the third day failed to reduce any of the SR properties examined. With the exception of \(V_{\text{max}}\), which recovered to preexercise levels, the failure to find an exercise effect occurred in the presence of incomplete recovery. Although Ca\(^{2+}\) release recovered to levels observed before exercise at day 1, during the first day of recovery, Ca\(^{2+}\) uptake was more delayed and did not recover until day 2. The consecutive days of exercise also increased SERCA1a expression, an adaptation not evident until recovery day 2. The differences in time-course responses in the different SR properties examined emphasizes the limitation of examining acute and/or adaptive responses of the SR to exercise at the same time point. In a related study, we examined the effects of the consecutive-day protocol of exercise and recovery on mechanical function (53). As expected, we have observed that the first and final days of exercise resulted in a pronounced reduction in vastus lateralis force at low frequencies of electrical stimulation, with recovery observed between days. However, after three consecutive days of exercise, incomplete recovery of force was observed at both high and low frequencies of stimulation during all 3 recovery days. The reduction in force at low frequencies of stimulation has been labeled low-frequency fatigue (LFF) (15), with post-contraction depression (PCD) used to indicate the existence of fatigue at both low and high frequencies of stimulation during all 3 recovery days. The reduction in force at low frequencies of stimulation has been labeled low-frequency fatigue (LFF) (15), with post-contraction depression (PCD) used to indicate the existence of fatigue at both low and high frequencies of stimulation (5). Force loss under these conditions has been shown to persist for hours and even days (5, 15) and appears to be due to reductions in [Ca\(^{2+}\)], possibly as a direct result of disturbances in SR Ca\(^{2+}\) cycling (1). It is possible that mechanisms that we have observed in Ca\(^{2+}\) release and Ca\(^{2+}\) uptake on the first and third
day of exercise may be involved in LFF. However, the persistence of fatigue during the recovery days while SR Ca\(^{2+}\)-cycling properties have recovered to levels not different from preexercise on day 1 indicates that other factors are involved.

**GRANTS**

Financial support for this study was received from the Natural Sciences and Engineering Council of Canada (H. J. Green).

**REFERENCES**


