Muscle sarcoplasmic reticulum Ca\(^{2+}\) cycling adaptations during 16 h of heavy intermittent cycle exercise


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REPEATED MUSCLE CONTRACTIONS are known to result in neuromuscular fatigue and weakness. When assessed under standardized conditions, fatigue is normally defined as a reduction in force generation immediately after the exercise (17, 40), whereas weakness refers to the delayed recovery of force (9, 14). Both muscle weakness and fatigue may be manipulated during repetitive activity by varying the intensity and duration of each work bout as well as the length of recovery between work bouts (7). By altering the characteristics of the work and recovery cycles, it is possible to selectively challenge the excitation and contraction and metabolic processes in the working muscles and to investigate their role in the weakness and fatigue that are observed. One such model that we have employed previously involves a 6-min period of heavy dynamic exercise followed by a 54-min period of recovery (21). With this protocol, a major activation of the metabolic pathways used to supply ATP occurs that is accompanied by large increases in the accumulation of metabolic by-products (4). The accumulation of the metabolic by-products is believed to disturb one or more of the processes involved in muscle contractile function (19). The 54 min of recovery between exercise bouts allow for restoration of muscle phosphorylation potential and metabolic by-product concentration (6, 33). Continuously repeating this protocol provides the opportunity to examine the time-course adaptations both in muscle mechanical function and in the behavior of the excitation-contraction and metabolic processes. In previous work from our group, we have used this model, which we extended to 16 h to investigate selected adaptations but only in response to a standardized exercise task performed in the days before and after the intermittent protocol. We have observed that, under these conditions, pronounced adaptations occur in the muscle metabolic responses (21) and in sarcoplasmic reticulum function to exercise (43). Unclear are the adaptations that occur at different time points during the intermittent protocol itself. In a recent study, we have repeated the same identical intermittent exercise protocol and investigated a variety of properties during the exercise itself.

One such area of interest was to determine the effects of repetitive activity on muscle fatigue and weakness by performing serial measurements of muscle mechanical function during 16 h of heavy intermittent cycle exercise performed for 6 min once per hour (20). Immediately after the first repetition of the exercise, fatigue was evident, as indicated by depressions in muscle quadriceps force observed over a wide range of stimulation frequencies (i.e., 10–100 Hz). The initial repetition of the exercise also resulted in muscle weakness illustrated by a sustained loss of force during the 54-min period of recovery before the second repetition of the exercise.

Unexpectedly, we have found that as the number of repetitions of the exercise progressed, both fatigue and weakness were attenuated. Before the 15th bout, as an example, weakness was present but only at low frequencies of stimulation (10 and 20 Hz) and not at high frequencies of stimulation (50 and 100 Hz). No fatigue regardless of stimulation frequency was observed with the 15th repetition. At question is the underlying cellular site(s) responsible for the mechanical events observed with the intermittent exercise protocol. To address this issue, we have also extracted tissue samples from the vastus lateralis at different time points to investigate the cellular responses.

Although all of the excitation and contraction processes in muscle may be implicated in the fatigue and weakness patterns...
that were observed, the regulation of the free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) transient by the sarcoplasmic reticulum (SR) remains particularly inviting (47). Measurements of intracellular [Ca\(^{2+}\)] during repetitive stimulation in single muscle fibers of the mouse have clearly demonstrated a reduction in [Ca\(^{2+}\)]; during the latter stages of a relatively brief protocol when force is substantially compromised (1). The reduction in [Ca\(^{2+}\)] could explain the depression in force that we have observed at the different frequencies of stimulation after the first repetition of the exercise and during recovery. Moreover, improvement in [Ca\(^{2+}\)] may also explain the protection that occurred in mechanical function as the number of repetitions of the exercise increased. The depression in force observed at low frequencies of stimulation, as an example, which is known to persist for hours (14), is mediated, in large part, by reductions in [Ca\(^{2+}\)] (1, 3). At high frequencies of stimulation, force may or may not be compromised, depending on the magnitude of change in [Ca\(^{2+}\)]; given the asymptotic nature of the force-[Ca\(^{2+}\)] curve (11).

Exercise-induced disturbances in Ca\(^{2+}\) handling may be secondary to direct alterations in SR function and not to more central processes involved in signal transmission to the SR. Previous work by our group (12, 43, 44) and others (8, 25) on humans has shown that repetitive exercise induces pronounced reductions in SR Ca\(^{2+}\) uptake and Ca\(^{2+}\) release when assessed in vitro in homogenates prepared from tissue extracted from human muscle. The reductions in both Ca\(^{2+}\) release and Ca\(^{2+}\) uptake appear to occur as a consequence of site-specific structural alterations to the calcium release channel and the Ca\(^{2+}\)-ATPase proteins, respectively (16, 26). Allen et al. (1) have shown that in repetitively stimulated mouse muscle the reduction in [Ca\(^{2+}\)] that occurs is mediated primarily by a reduction in Ca\(^{2+}\) release. The reduction in Ca\(^{2+}\) release persists for a sustained period after the exercise and has been mechanistically linked to low-frequency fatigue (1, 9).

The purpose of this study was to investigate the effects of repeated bouts of heavy intermittent exercise on muscle SR Ca\(^{2+}\) handling as assessed in vitro. We have hypothesized that initial repetition of the exercise would result in large reductions in both SR Ca\(^{2+}\) uptake and Ca\(^{2+}\) release and that those reductions would persist during the recovery period. As the number of repetitions increased, less of a disturbance would be observed in these processes both with exercise and after recovery. In the case of Ca\(^{2+}\) uptake, the reductions can be explained by reductions in the maximal activity of the Ca\(^{2+}\)-ATPase that occur independent of changes in Ca\(^{2+}\) sensitivity or in the ratio of the Ca\(^{2+}\) uptake to Ca\(^{2+}\)-ATPase activity, defined as the apparent coupling ratio.

**METHODS**

**Participants.** Twelve untrained but healthy volunteers (9 men and 3 women) participated in the study. The mean age, height, and mass were 21.8 ± 0.86 yr, 173 ± 2 cm, and 70.2 ± 3.6 kg, respectively. Peak aerobic power (V\(_{\text{O2 peak}}\)), assessed during progressive cycle exercise to fatigue, was 3.07 ± 0.17 l/min. As a condition of entry into the study, participants could not be engaged in vigorous exercise on a regular basis. As required, all participants were fully informed of all experimental procedures and all associated risks before written consent was obtained. Written approval for the research was granted by the Office of Research Ethics at the University of Waterloo.

**Experimental design.** The basic protocol employed to investigate changes in muscle SR responses involved 6 min of cycle exercise performed at ~91% V\(_{\text{O2 peak}}\), once per hour for 16 h. Tissue samples were extracted from the vastus lateralis muscle before exercise (B) and immediately after exercise (A) at repetition 1 (R1), repetition 2 (R2), repetition 9 (R9), and repetition 16 (R16). Given the number of biopsies involved, it was necessary for the participants to perform the intermittent exercise on two separate occasions, separated by ~4 wk. On one occasion, only the first two repetitions (R1 and R2) were performed, with tissue extracted before and after each repetition. On a second occasion, the entire 16-h protocol was completed, with tissue extracted before and after R9 and R16. The order of conditions was randomly assigned. Given the limited number of biopsies that could be performed per individual, a decision had to be made regarding the specific repetitions to be sampled. The strategy selected was to distribute the sampling over the number of repetitions while allowing an emphasis on the early response (R1 and R2).

On a given experimental day, the participants reported to the laboratory at ~7 AM. The intermittent exercise protocol was initiated at 9 AM. In the interval between each exercise bout, the volunteers underwent general preparation for the experiment, which also involved tissue sampling. This included cleansing (alcohol) the biopsy sites, freezing (2% xylocaine), and making a small incision. Four separate sites were used for tissue sampling on a given day, two from each leg with the order of sites used for the biopsies randomized. These same procedures were also followed during the second experimental session. The limited region of the vastus lateralis selected for tissue samples was based on early study (5), which has become the standard used by many laboratories. Care was taken to control for the depth of the sample.

During R1, R2, R7, and R15, respiratory gas collection for measurement of oxygen consumption (V\(_{\text{O2}}\)) was performed before the exercise and continuously throughout the exercise according to previous methods published by our group (23). The V\(_{\text{O2}}\) was calculated for each minute of the exercise and used to assess the effects of the repetitions on changes in oxidative phosphorylation. An electronically braked cycle ergometer (Siemens Elma 380B), calibrated periodically throughout each experimental day, was used for all tests. Seat height was individually adjusted and standardized for each repetition of the exercise. All exercise sessions were performed at a pedaling rate of 60 rpm. Individual workloads were established to result in a V\(_{\text{O2}}\) response (5–6 min of work bout) of ~90% V\(_{\text{O2 peak}}\). The V\(_{\text{O2 peak}}\) of each participant was assessed at least 2 wk before the first experimental day. The temperature and relative humidity of the laboratory ranged between 20 and 22°C and 39 and 48%, respectively.

In addition to the respiratory gas-exchange measurements and tissue sampling, we also measured the mechanical properties of the quadriceps muscle. These measurements were performed before and after R1, R2, R4, R7, R12, and R15. These results have been reported elsewhere (20).

Before reporting to the laboratory, the volunteers were required to ingest an Ensure (250 kcal) meal replacement consisting of 9.4 g of proteins, 6.7 g of fat, and 38 g of carbohydrates (Ross Products Division, Saint-Laurent, Quebec, Canada). During the 16-h intermittent exercise protocol, water (room temperature) was allowed ad libitum after the first 2 h. At this time the volunteers were also allowed to consume selected vegetables, fruits, and Gatorade bars on a regular basis. Dietary composition and energy intake during different segments of the intermittent protocol are reported in our laboratory’s earlier paper (20). During the 54-min period between each exercise repetition, volunteers remained in the test area and, with the exception of the mechanical function tests, remained inactive.

**SR properties.** For measurement of SR Ca\(^{2+}\) cycling properties, a portion of the tissue sample was immediately homogenized and frozen in liquid N\(_2\). A second portion of the tissue was placed directly in liquid N\(_2\). All samples were stored at ~80°C until analyses. The SR Ca\(^{2+}\) handling properties assessed included Ca\(^{2+}\) uptake and Ca\(^{2+}\)...
release, which were determined as part of the same assay, and Ca$^{2+}$-ATPase activity. All SR functional measurements were performed on crude homogenates. Because these measurements have been described in detail elsewhere (12, 36), only a brief description is provided here.

Homogenates were prepared with 30–40 mg of tissue diluted 11:1 (vol/wt) in an ice-cold buffer containing (in mM) 250 sucrose, 5 HEPES, 10 Na$_2$S, and 0.2 PMSF (pH 7.5) by using a handheld glass homogenizer (Duall 20, Kontes). Before freezing, the homogenates were separated into multiple aliquots, which were used for the assay of specific properties. On a given analytical day, aliquots were thawed and processed for the duplicate measurements of a specific property. For a given participant the measurement of all samples for a given property was assessed in the same analytical session.

Ca$^{2+}$-ATPase activity. Ca$^{2+}$-dependent ATPase activity was measured at 37°C by using a spectrophotometric method originally developed for homogenates by Simonides and van Hardeveld (38). The reaction medium contained (in mM) 20 KCl, 20 HEPES, 15 MgCl$_2$, 1 EGTA, 10 Na$_2$S, 5 ATP, and 10 phosphoenolpyruvate, pH 7.0. Just before the reaction was started, 18 U/ml lactate dehydrogenase, 18 U/ml pyruvate kinase, 0.3 mM NADH, and 25 μl homogenate were added to a cuvette containing 1 ml of reaction buffer. 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 inhibits Ca$^{2+}$-ATPase activity (38). By measuring Ca$^{2+}$-ATPase activity with and without the ionophore A-23187, it is possible to gain an indirect assessment of the effects of exercise on changes in SR membrane integrity.

The three kinetic properties of the enzyme that were measured were 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% of $V_{\text{max}}$.

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, which corresponds to the contamination of other membrane ATPases in the homogenate, was determined by using 40 μM of the Ca$^{2+}$-ATPase inhibitor cyclopiazonic acid (CPA) (37). Ca$^{2+}$-ATPase activity is based on the difference between total ATPase activity measured without CPA and the basal ATPase measured with CPA. All spectrophotometric assays were performed in duplicate at 340 nm (Shimadzu UV 160) using 25 μl of homogenate (~2 mg tissue per assay). Measurement of [Ca$^{2+}$]$_r$, which is used to assess Ca$^{2+}$ dependency of the Ca$^{2+}$-ATPase reaction, was performed with dual-wave spectrophotometry and the Ca$^{2+}$-fluorescent dye indo-1, as previously detailed (42, 43). The excitation wavelength was 355 nm, and the emission was measured at 405 and 485 nm for Ca$^{2+}$-bound indo-1 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.

Ca$^{2+}$ uptake and Ca$^{2+}$ release. Ca$^{2+}$ uptake and Ca$^{2+}$ release rates were measured in duplicate during the same assay at 37°C in a reaction buffer containing (in mM) 200 KCl, 20 HEPES, 15 MgCl$_2$, 10 Na$_2$S, 0.005 Na$_2$N$_2$N$’$-tetraakis(2-pyridylimethyl)-ethylenediamine, 5 oxalate, and 10 phosphoenolpyruvate. Before each assay, 1.5 μM indo-1, 18 U/ml lactate dehydrogenase and 18 U/ml pyruvate kinase were added to 2 ml of reaction buffer. In addition, 2.5 μl of CaCl$_2$ (10 mM) were added to the cuvette to produce a constant starting [Ca$^{2+}$]$_r$ of ~3.5 μM. Ca$^{2+}$ uptake was initiated by adding 5 mM ATP. Ca$^{2+}$ uptake rates were determined at four [Ca$^{2+}$]$_r$ concentrations (500, 1,000, 1,500, and 2,000 nM) by differentiating a linear fit curve. [Ca$^{2+}$]$_r$ was measured by use of the fluorescent dye indo-1, as earlier described. Additional assay description is provided in earlier publications by our group (13, 42, 43).

To assess the relationship between Ca$^{2+}$ uptake (2,000 nM) and $V_{\text{max}}$, we calculated the ratio between these two properties. We have called this the apparent coupling ratio because the ratio was not determined under identical assay conditions. Performing the Ca$^{2+}$ uptake and Ca$^{2+}$-ATPase measurements under identical conditions is not possible given the need to use a precipitating agent for Ca$^{2+}$ uptake and a membrane Ca$^{2+}$-permeabilizing agent for Ca$^{2+}$-ATPase activity. Moreover, given the limited sensitivity of indo-1 for Ca$^{2+}$, it is not clear whether maximal Ca$^{2+}$ uptake was obtained at a [Ca$^{2+}$]$_r$ of 2,000 nM.

Ca$^{2+}$ release was initiated after Ca$^{2+}$ uptake had stabilized using 20 μM of 4-chloro-m-cresol (4-CMC). We used 4-CMC as the releasing agent on the basis of a previous study from our laboratory that demonstrated that AgNO$_3$, a common agent in Ca$^{2+}$ release determinations, causes reversal of the Ca$^{2+}$-ATPase pump (42). We also measured two phases of Ca$^{2+}$ release, namely phase 1 and phase 2. In our hands, we find that 4-CMC induces a biphasic Ca$^{2+}$ release, namely a rapid early phase (phase 1) and a more delayed, slower phase (phase 2) (42). Maximal release rates for each phase were calculated by using the same method as for Ca$^{2+}$ uptake and differentiating a linear fit curve.

For all SR properties, protein was determined in duplicate by the method of Lowry as modified by Schacterle and Pollack (35).

Data analyses. To determine the effects of time (number of repetitions) and exercise (B vs. A), two-way ANOVA procedures for repeated measurements were employed using the subject pool as a single block. In a preliminary analysis, we examined for differences in responses between genders and observed similar trends, suggesting that no bias existed. Where significance was found, the Newman-Keuls technique was applied to determine which means were significantly different. Statistical significance was accepted at $P < 0.05$. Data are represented as means ± SE. Throughout the text, statistical differences between means are indicated only as “differences” based on the level of probability established.

RESULTS

$\dot{V}_O_2$. To assess whether changes in oxidative phosphorylation occurred during the repetitions of the exercise, we measured the $\dot{V}_O_2$ responses during each minute of the exercise (Table 1). For all repetitions except R7, progressive increases in $\dot{V}_O_2$ were observed during the first 3 min of exercise. For R7, the increases only occurred during the first 2 min of exercise. For R1 and R15 but not R2 and R7, further increases were not observed until the sixth minute of exercise. As the number of repetitions increased, elevations in $\dot{V}_O_2$ were observed particularly during the early stages of the exercise and early in the protocol after the first two repetitions. At R7, for example, $\dot{V}_O_2$ was greater than R1 for the first 4 min of the exercise. At R15, $\dot{V}_O_2$ was elevated over both R1 and R2 during each minute of the exercise with the exception of the fifth. At the sixth minute, $\dot{V}_O_2$ exceeded R1, R2, and R7.

SR properties. The initial 6 min of cycling exercise (R1) resulted in an approximate 26% reduction in $V_{\text{max}}$ (Fig. 1). Although some recovery was evident in the 54-min period before R2, $V_{\text{max}}$ still remained depressed by 17%. Before exercise at R9 and R16, $V_{\text{max}}$ was not different than observed before exercise at R1. Exercise failed to induce changes in $V_{\text{max}}$ at either R2 or R9. However, at R16 a 12% reduction in $V_{\text{max}}$ was observed. After exercise $V_{\text{max}}$ was higher than R1 at all of the repetitions studied, namely R2, R9, and R16. The changes in $V_{\text{max}}$ were not accompanied by changes in the other kinetic properties of the enzyme examined, specifically Ca$^{50}$.
Table 1. Effects of 16 h of intermittent exercise on oxygen consumption

<table>
<thead>
<tr>
<th>Time, min</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>V\textsubscript{O2}, l/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>1.34 ± 0.09</td>
<td>2.22 ± 0.13\textsuperscript{a}</td>
<td>2.40 ± 0.14\textsuperscript{b}</td>
<td>2.57 ± 0.14\textsuperscript{b}</td>
<td>2.73 ± 0.17\textsuperscript{b}</td>
<td>2.82 ± 0.18\textsuperscript{b,c,d}</td>
</tr>
<tr>
<td>R2</td>
<td>1.35 ± 0.08</td>
<td>2.33 ± 0.12\textsuperscript{a}</td>
<td>2.51 ± 0.13\textsuperscript{b}</td>
<td>2.61 ± 0.14\textsuperscript{b}</td>
<td>2.76 ± 0.15\textsuperscript{b,c}</td>
<td>2.76 ± 0.17\textsuperscript{b,c}</td>
</tr>
<tr>
<td>R7</td>
<td>1.45 ± 0.10\textsuperscript{a}</td>
<td>2.50 ± 0.15\textsuperscript{a,b}</td>
<td>2.67 ± 0.16\textsuperscript{a,b}</td>
<td>2.76 ± 0.16\textsuperscript{a,b}</td>
<td>2.87 ± 0.18\textsuperscript{b,a}</td>
<td>2.96 ± 0.18\textsuperscript{b,a}</td>
</tr>
<tr>
<td>R15</td>
<td>1.55 ± 0.08\textsuperscript{a}</td>
<td>2.56 ± 0.13\textsuperscript{a,b}</td>
<td>2.85 ± 0.15\textsuperscript{a,b,g}</td>
<td>2.89 ± 0.18\textsuperscript{a,b,f,g}</td>
<td>2.88 ± 0.19\textsuperscript{b}</td>
<td>3.05 ± 0.17\textsuperscript{b,c,d,f}</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 11). V\textsubscript{O2}, oxygen consumption; R1, R2, R7, R15, number of repetitions of the exercise: \textsuperscript{a}Significantly different (P < 0.05) from 1 min. \textsuperscript{b}Significantly different (P < 0.05) from 2 min. \textsuperscript{c}Significantly different (P < 0.05) from 3 min. \textsuperscript{d}Significantly different (P < 0.05) from 4 min. \textsuperscript{e}Significantly different (P < 0.05) from 5 min. \textsuperscript{f}Significantly different (P < 0.05) from R1. \textsuperscript{g}Significantly different (P < 0.05) from R2. \textsuperscript{h}Significantly different (P < 0.05) from R7.

and n\textsubscript{H}, either before or after exercise (Table 2). In addition, the ionophore ratio, a measure of the changes in membrane permeability to Ca\textsuperscript{2+}, was unaffected by the intermittent exercise protocol (Fig. 2).

In general, the changes in Ca\textsuperscript{2+} uptake paralleled the changes in V\textsubscript{max} (Fig. 3). At [Ca\textsuperscript{2+}]\textsubscript{i} of 2,000 nM, for example, reductions in Ca\textsuperscript{2+} uptake with exercise only occurred at R1 and R16. Full recovery of Ca\textsuperscript{2+} uptake was observed before exercise at R9 and R16 but not at R2. After exercise, Ca\textsuperscript{2+} uptake was higher at both R9 and R16 compared with R1. Although the same trends were observed at the lower [Ca\textsuperscript{2+}]\textsubscript{i} used to measure Ca\textsuperscript{2+} uptake, namely 1,500, 1,000, and 500 nM, significant differences both after exercise and after recovery were not as extensive (data not presented). Apparent coupling ratios, operationally defined as the ratio between Ca\textsuperscript{2+} uptake measured at 2,000 nM and V\textsubscript{max}, were not altered regardless of the number of repetitions performed (Fig. 4).

We found the Ca\textsuperscript{2+} release was modified by the exercise, which was repetition dependent (Fig. 5). At R1, phase 1 Ca\textsuperscript{2+} release was reduced by 32% after exercise. No changes were observed with exercise at R2, R9, or R16. Phase 1 Ca\textsuperscript{2+} release was not different before exercise between R1 and R2.

The changes in phase 2 Ca\textsuperscript{2+} release were the same as observed for phase 1. Phase 1 Ca\textsuperscript{2+} release was higher after exercise at R9 and R16 than at R1 and R2. For phase 2 Ca\textsuperscript{2+} release, higher values were observed after exercise at R16 compared with after exercise at R1.

DISCUSSION

Our 16-h intermittent work protocol has uncovered several novel features of the SR Ca\textsuperscript{2+}-handling behavior assessed in vitro not previously recognized. As hypothesized, we found that Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+} release, both phase 1 and phase 2, were depressed after the first repetition of the exercise. With additional repetitions of the exercise the responses depended both on the property in question and the number of repetitions completed. In the case of Ca\textsuperscript{2+} uptake, a progression of responses was observed, including a failure of the exercise to induce a decrease in Ca\textsuperscript{2+} uptake with normal preexercise levels (R9), and a blunted reduction in Ca\textsuperscript{2+} uptake with normal preexercise levels (R16). For Ca\textsuperscript{2+} release, depressions in both phase 1 and phase 2 occurred at R1. For R2 and all subsequent repetitions that were examined, exercise failed to alter either phase of Ca\textsuperscript{2+} release. For phase 1 and phase 2 Ca\textsuperscript{2+} release, significant depressions were not observed before exercise after R1 at any of the repetitions examined.

The adaptations that we have described in SR Ca\textsuperscript{2+} cycling properties in human muscle with the increasing number of repetitions have implications for the regulation of Ca\textsuperscript{2+} release during exercise and contraction. The progressive reduction in Ca\textsuperscript{2+} release with increasing number of repetitions may be related to the ability of the SR to handle the increased Ca\textsuperscript{2+} flux generated by exercise.

Table 2. Effects of 16 h of intermittent exercise on kinetic properties of the Ca\textsuperscript{2+}-ATPase

<table>
<thead>
<tr>
<th>Repetition</th>
<th>R1</th>
<th>R2</th>
<th>R9</th>
<th>R16</th>
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<tbody>
<tr>
<td>Ca\textsubscript{50}, nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1,094 ± 92</td>
<td>1,252 ± 201</td>
<td>1,453 ± 219</td>
<td>1,337 ± 145</td>
</tr>
<tr>
<td>A</td>
<td>1,562 ± 177</td>
<td>1,300 ± 70</td>
<td>1,495 ± 175</td>
<td>1,441 ± 134</td>
</tr>
<tr>
<td>n\textsubscript{H}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2.03 ± 0.06</td>
<td>2.05 ± 0.06</td>
<td>1.99 ± 0.04</td>
<td>1.99 ± 0.04</td>
</tr>
<tr>
<td>A</td>
<td>2.05 ± 0.03</td>
<td>2.08 ± 0.08</td>
<td>2.04 ± 0.06</td>
<td>2.02 ± 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 12). R1, R2, R9, R16, number of repetitions of the exercise session; Ca\textsubscript{50}, Ca\textsuperscript{2+} concentration need to elicit 50% maximal Ca\textsuperscript{2+}-ATPase activity, n\textsubscript{H}, Hill coefficient defined as determined from Hill plots by using the relationship between free Ca\textsuperscript{2+} concentration and Ca\textsuperscript{2+}-ATPase activity and the section of the curve that corresponded to 10–90% of the maximal Ca\textsuperscript{2+}-ATPase activity, B, before exercise, A, after exercise. No significant differences were observed either for exercise or the number of repetitions (P > 0.05) either Ca\textsubscript{50} or n\textsubscript{H}.
Repetitions of the exercise are particularly novel. As expected, Ca$^{2+}$ uptake was depressed during the first repetition of the exercise (18, 25, 29, 43, 44). In addition, the reductions in $V_{\text{max}}$ at R1 are as expected and appear to explain the reductions in Ca$^{2+}$ uptake (10, 25, 29, 43). The changes in $V_{\text{max}}$ at R1 occurred in the absence of changes in the other kinetic properties of the enzyme, namely Ca$_{50}$ and $n_{\text{H}}$, or in the ionophore ratio, an indirect measure of membrane integrity (28). The changes in $V_{\text{max}}$ with exercise have been attributed to structural alterations in the region of the nucleotide binding site of the enzyme (26). It is clear that these changes only impact the maximal catalytic activity of the enzyme and not the apparent affinity of the enzyme for [Ca$^{2+}$]$_{c}$ in the absence of changes in the other kinetic properties of the enzyme. The behavior in Ca$^{2+}$ uptake (2,000 nM) observed during the remainder of the exercise protocol both after the inactive period and during the exercise itself also can be explained by the response in $V_{\text{max}}$. Accordingly, the incomplete recovery observed at R2 for Ca$^{2+}$ uptake followed by the recovery and subsequent depression in Ca$^{2+}$ uptake at R16 was similar to $V_{\text{max}}$. As with R1, Ca$_{50}$ and $n_{\text{H}}$, as well as the ionophore ratio, remained unaltered.

The failure of Ca$^{2+}$-ATPase and Ca$^{2+}$ uptake to recover before exercise at R2 was also as expected given the protracted time necessary to restore normal mechanical function after contractile activity (14). At R2 and R9, exercise failed to effect reductions in $V_{\text{max}}$ and in Ca$^{2+}$ uptake. However, at these time points, we found that full recovery of these properties to the level observed before R1 had not occurred. By R16, full recovery of $V_{\text{max}}$ and Ca$^{2+}$ uptake had resulted, which interestingly was again accompanied by reductions in both properties with exercise, albeit at an attenuated level.

The failure of $V_{\text{max}}$ to be reduced with exercise while in the depressed state is a novel observation, which could suggest that a given subgroup of the enzyme is only susceptible to modification with exercise and/or that related adaptations in the intracellular environment occur to protect further deterioration in enzyme function. This could occur as a result of reductions in free radical accumulation, thought to be a major factor in the structural alterations that occur in the region of the adenine nucleotide site (24). Alternatively, mobilization of heat shock proteins (HSP) such as HSP70, known to be rapidly induced (31), could protect enzyme integrity. We have recently shown that HSP70 can migrate to the SR Ca$^{2+}$-ATPase and protect enzyme function in vitro during temperature stress (41). One or more of these mechanisms could also be operating at R16, given the attenuated decrease that occurs in Ca$^{2+}$-sequestering properties with the exercise. Interestingly, when full recovery of the maximal catalytic activity of the enzyme occurs, the exercise-induced effects are again observed.

The complex behavior of the Ca$^{2+}$-ATPase activity was emphasized in a previous study by our group employing the 16-h intermittent exercise model (43). In that study, we measured Ca$^{2+}$-ATPase activity and Ca$^{2+}$ uptake before the intermittent protocol using a two-stage cycling test. As expected, both properties decreased with exercise. When the same cycling task was repeated 36–48 h after the intermittent exercise, Ca$^{2+}$-ATPase activity and Ca$^{2+}$ uptake, although decreased at rest, increased during the two-step exercise protocol.

Repetition-dependent adaptations to Ca$^{2+}$ release were also observed to occur in the present study. We assessed kinetic phases of Ca$^{2+}$ release, namely phase 1 and phase 2, on the basis of our previous work, which shows a clear demarcation between phases (42). Phase 1, the more rapid and pronounced phase, was depressed with exercise but only at R1. As the number of repetitions of the exercise increased, phase 1 Ca$^{2+}$ release was restored during recovery and exercise failed to have an effect. In the case of phase 2 Ca$^{2+}$ release, we found a similar response to that observed for phase 1 Ca$^{2+}$ release. We conclude that, as with Ca$^{2+}$ sequestration, Ca$^{2+}$ release also showed an adaptive response to the repetitive bouts of exercise.

As observed with the Ca$^{2+}$-ATPase enzyme, Ca$^{2+}$ has been previously shown to result in structural adaptations to the ryanodine receptor (RyR) (16, 32). Moreover, repair of the
The RyR or the synthesis of new RyR also appears to take a considerable period of time (1). Our findings indicate that, whatever the mechanism, the normalization of Ca^{2+}/H^{+} release during recovery occurs relatively early in the intermittent protocol. Unlike the Ca^{2+}/H^{+}-ATPase, restoration of function is accompanied by full protection of the RyR during the exercise. This is particularly evident for phase 1 Ca^{2+}/H^{+} release. The mechanism by which this protection occurs is unclear but may involve, as with the Ca^{2+}/H^{+}-ATPase, a reduction in the accumulation of free radicals and/or the mobilization of HSPs (15).

Our results for Ca^{2+} uptake and Ca^{2+}/H^{+}-ATPase activity generally paralleled each other, implying that the modifications in Ca^{2+} uptake are primarily dependent on changes in Ca^{2+}/H^{+}-ATPase activity. This suggests that the efficiency of Ca^{2+} transport was not modified regardless of the [Ca^{2+}]_{i}. The changes that we have observed in SR Ca^{2+} cycling properties may be linked to the mechanical fatigue and weakness that we have described (20). After the first repetition of the exercise, we observed reductions in force of the quadriceps at a variety of stimulation frequencies in conjunction with a prolongation of the maximal rates of force development and relaxation. Here we show that these changes were associated with reductions in both Ca^{2+} uptake and Ca^{2+} release observed during the first repetition of exercise. Decreases in Ca^{2+} release kinetics are believed to reduce the rate at which force is generated by reducing the kinetics of weak to strong actomyosin formation (30), whereas decreases in the rate of which relaxation occurs have been partially explained by decreases in Ca^{2+} uptake (46, 48). Allen et al. (1) have observed in mouse muscle fibers that the fatigue observed correlates with reductions in the [Ca^{2+}]_{i} transient that occurs secondary to reductions in Ca^{2+} release. These investigators have also found reductions in the kinetics of Ca^{2+} uptake (1).

After the first repetition of the exercise, the relationship between SR Ca^{2+} cycling behavior and mechanical function of the quadriceps became less clear. During the inactive period between R1 and R2, force failed to recover regardless of stimulation frequency. A similar finding was also observed for the maximal rates of force development and relaxation. During this period, we have also observed an incomplete recovery of Ca^{2+} uptake. Both phase 1 and phase 2 Ca^{2+} release also appear reduced during this period, but the differences were not significant. As the number of exercise repetitions increased, mechanical function returned toward preexperimental levels during the recovery periods and showed no effects of the exercise itself. Interestingly, during this period, Ca^{2+} uptake and Ca^{2+} release also returned toward normal and showed no effects of exercise. At R16, exercise resulted only in a reduction in Ca^{2+} uptake. The reduction in Ca^{2+} uptake, which was considerably smaller than observed at R1, was not accompanied by changes in any of the mechanical properties examined. These results suggest that the adaptations that we have observed in the SR Ca^{2+} handling during the repetitive exercise protocol were associated with the attenuation in fatigue and...
weakness that we reported. However, to implicate changes in SR Ca\(^{2+}\) handling, measurements of [Ca\(^{2+}\)]\(_{i}\)) are required. Experiments on intact single-fiber preparations have repeatedly documented that the fatigue observed with repetitive tetanic contractions occurs in conjunction with a pronounced reduction in the [Ca\(^{2+}\)]\(_i\) transient (1). The reduction in the [Ca\(^{2+}\)]\(_i\) transient appears to be mediated primarily by a reduction in SR Ca\(^{2+}\) release (1). At present it is unclear whether the reduction in Ca\(^{2+}\) release is mediated by the RyR directly (2) or is secondary to a more central process. Because membrane excitability appears normal (49), a defect in signal transmission between the T tubule and RyR has been hypothesized (39). Our study suggests that at least at R1 structural modifications to the RyR occurred, as has been previously shown (16, 32). This may explain the reductions in both phases of Ca\(^{2+}\) release measured in vitro under supposedly optimal conditions.

At low frequencies of stimulation, the reduction in force observed at R1 generally persisted throughout the protocol. Beyond R1, exercise was without effect in causing a greater reduction in force. This form of weakness is typical of low-frequency fatigue (9). Single-fiber studies have shown this type of weakness to be closely associated with decreases in the [Ca\(^{2+}\)]\(_i\) transient secondary to reduced Ca\(^{2+}\) release (1). During this period, we could find no depression in phase 1 or phase 2 Ca\(^{2+}\) release either before or after exercise. The dissociation that we have shown between weakness and Ca\(^{2+}\) release, both phase 1 and phase 2, suggests that structural alterations cannot explain the apparent reduction in Ca\(^{2+}\) release that occurs in the intact fiber preparation during this period.

Also of interest is the functional significance of the decrease in Ca\(^{2+}\) uptake observed during the experimental protocol. Decreases in Ca\(^{2+}\) uptake could have at least two effects in influencing Ca\(^{2+}\) release. On the one hand, it could help stabilize the [Ca\(^{2+}\)]\(_i\) transient given the reduction in Ca\(^{2+}\) release that occurs. On the other hand, decreases in Ca\(^{2+}\) uptake could reduce Ca\(^{2+}\) loading into the SR and compromise Ca\(^{2+}\) release. More recent work, again employing intact single fibers, has found evidence that depressions in Ca\(^{2+}\) uptake can help explain the reductions in Ca\(^{2+}\) release (1). However, as with Ca\(^{2+}\) release, we have found that Ca\(^{2+}\) uptake is normalized, whereas low-frequency fatigue persists. It is possible that low-frequency fatigue could have been even more pronounced if not for the reduced rate of relaxation. Given that unfused tetani occur at low frequencies of stimulation, a prolongation of relaxation rate would result in higher force levels between stimuli and consequently higher peak forces (45). Because the depression in the maximal rate of relaxation persisted at low stimulation frequencies despite normal Ca\(^{2+}\) uptake in our study, it would appear that events involving Ca\(^{2+}\) dissociation from the thin filament can also influence relaxation rates (46, 48). It is also conceivable that the myofibrillar proteins display a decreased sensitivity to Ca\(^{2+}\), resulting in a reduced mechanical response at different stages of the repetitive cycle exercise (22, 27).

The possibility remains that the sequence of mechanical changes observed with regard to both fatigue and weakness is under complex regulatory control, the etiology of which may change during the course of a protracted session of heavy intermittent exercise.

An important issue is the degree to which our changes in Ca\(^{2+}\)-handling behavior observed over the course of the repetitive exercise corresponds with the changes in fatigue and weakness that we have observed (20). These comparisons must be put in context. We have used the intermittent exercise protocol both to challenge SR Ca\(^{2+}\) cycling behavior and to induce fatigue. By necessity, fatigue was measured 4–5 min after the exercise, the time needed to position the volunteer on the testing chair and to prepare for the measurements. Because our measurements of the SR properties were measured in vitro under optimal conditions on tissue obtained either before or immediately after the exercise, the changes are thought to reflect acute regulatory factors and/or structural damage to the proteins. As such, the effects we have observed would be expected to be superimposed on the actual in vivo changes that reflect the disturbances that occur in the intracellular environment as well. With the heavy exercise bouts employed, the accumulation of metabolic by-products, known to affect SR Ca\(^{2+}\) uptake and Ca\(^{2+}\) release such as inorganic phosphate, free adenosine diphosphate, and hydrogen ions, would be expected to be substantial (21). Because the fatigue measurements were performed 4–5 min after the exercise, these metabolites, with the exception of hydrogen ions, were at preexercise levels (34). In the measurements after recovery, before the next exercise session, a period that involved in excess of 50 min, the metabolic profile would be at comparable to the rest state (34). For this reason the measurements of SR Ca\(^{2+}\) handling in vitro may offer some insight into the fatigue manifestations.

In summary, we have been able to demonstrate that when SR function is appropriately stressed with repeated bouts of heavy exercise, rapid adaptations occur. These adaptations result in less of a perturbation in Ca\(^{2+}\) uptake, which appears mediated by improved maintenance in Ca\(^{2+}\)-ATPase activity. In addition, disturbances in Ca\(^{2+}\) release, both phase 1 and phase 2, were eliminated during the 16-h protocol. Collectively, these findings suggest improved Ca\(^{2+}\) cycling behavior in repetitively exercised muscle and improved fatigue resistance.

GRANTS

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