Effects of varied fatigue protocols on sarcoplasmic reticulum calcium uptake and release rates

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Ward, Christopher W., Espen E. Spangenburg, Lillian M. Diss, and Jay H. Williams. Effects of varied fatigue protocols on sarcoplasmic reticulum calcium uptake and release rates. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R99–R104, 1998.—The purpose of this investigation was to examine changes in sarcoplasmic reticulum (SR) function in muscles subjected to different patterns of muscle activity. Frog sartorius muscles were stimulated with tetanic trains (100 ms, 100 Hz) delivered at rates of 2.0, 0.5, and 0.2 trains/s. In one set of experiments, stimulation was continued until force had declined to ∼17% of initial (constant fatigue), whereas in the other set, stimulation was continued for 1 min (constant duration). In the constant-fatigue experiments, Ca2+ uptake (1 mM MgATP) and release rates (25 μM AgNO3, 5 mM 4-chloro-m-cresol) were depressed by similar extents following each protocol. This occurred despite 1, 4, and 17 min of stimulation, respectively, used to induce fatigue. In the constant-duration experiments, larger reductions in SR function occurred following the highest frequency stimulation protocol. These data suggest that when muscles are fatigued to similar extents, depressions in SR function are independent of the activity protocol. On the other hand, when a constant duration of activity is imposed, changes in SR function are closely linked to the extent of force reduction.

frog sartorius muscle; indo 1; skeletal muscle

MUSCULAR ACTIVITIES requiring either short-term, high-intensity, or prolonged, low-intensity efforts typically result in progressive declines in the muscle's ability to generate force. This process, defined as fatigue, is thought to be a key factor limiting the individual's exercise and/or work capacity. Despite years of investigation, the precise mechanisms that mediate the fatigue process are not fully understood. Numerous investigators have provided a wealth of experimental evidence in an attempt to explain all or part of the fatigue process. In general, hypotheses concerning the mechanisms of fatigue fall into three broad categories: 1) depletion hypotheses that suggest that reduced availability of energy results in depressed force output, 2) accumulation hypotheses that propose that the build-up of metabolic by-products impairs the contractile process, and 3) calcium (Ca2+) exchange hypotheses that state that intrinsic alterations in the sarcoplasmic reticulum (SR) Ca2+ uptake and/or release properties limit activation of the contractile apparatus and reduce force output. Although proposed early by Eberstein and Sandow (8), this last hypothesis has recently gained increasing support. Much of this support comes from experiments that show that intracellular Ca2+ transients are depressed during the development of fatigue (1, 15, 19) and that the rates of SR Ca2+ release and uptake are reduced in skinned fibers and SR vesicles obtained from fatigued muscles (6, 9, 16, 17, 20, 21).

Without doubt, the fatigue process is complex, involving multiple factors and several mechanisms. The importance of each of these mechanisms likely depends on the type of muscle being studied, the type of activity employed, as well as model being used. Interestingly, a recent survey of pertinent literature indicates that in several different models using varied activity patterns, the rates of SR Ca2+ release and uptake are consistently depressed by 40–60% (21). For example, Williams et al. (22) found in frog muscle stimulated to fatigue within 3 min that Ca2+ uptake and release in skinned fibers were depressed, whereas Luckin et al. (16) found qualitatively similar results using rats and treadmill exercise lasting nearly 90 min. Studies such as these suggest that depressions in SR function are closely linked to the development of fatigue in a wide range of muscle activity patterns. That is, regardless of the type of activity used to induce fatigue, depressions in SR Ca2+ uptake and release occur and may contribute to the fatigue process.

Unfortunately, the disparate experimental approaches used and the differing degrees of fatigue elicited make it difficult to directly compare changes in SR Ca2+ uptake and release kinetics between varied activity patterns. Thus firm conclusions regarding the role of SR dysfunction in the fatigue process are somewhat tenuous. We suggest that if changes in SR function truly contribute to the fatigue process, then alterations should be apparent regardless of the mode of activity employed. With this in mind, the purpose of this investigation was to examine changes in SR function in muscles subjected to different stimulation patterns. In the first set of experiments, we used varying durations of stimulation, which resulted in similar degrees of fatigue. In the second set, we applied the stimulation protocols for similar durations, which resulted in varied degrees of fatigue. We then compared changes in SR Ca2+ uptake and release rates between protocols.

METHODS

In vitro muscle preparation. For all experiments, male grass frogs (Rana pipiens) were used and all procedures were approved by the Virginia Tech Animal Care and Use Committee. Animals were first placed in a cold-induced torpor (crushed ice-water slurry, 10 min). They were then rapidly decapitated, and both sartorius muscles were removed and placed in ice-cold Ringer solution.
The Ringer incubation solution contained (in mM) 115 NaCl, 2.5 KCl, 1.8 CaCl₂, 2.15 Na₂HPO₄, and 0.85 NaH₂PO₄ and was continually bubbled with room air (pH 7.2). Whole muscles were mounted in a temperature-controlled chamber (21°C), with one end of the muscle attached to a fixed post and the other to an isometric force transducer (Grass FT-03, 7P122 low-level direct current amplifier). Tetanic contractions were electrically evoked by 100-ms trains of pulses (0.2 ms, 100 Hz; Grass S-88 stimulator, SIU-S stimulus isolation unit) delivered across platinum wire electrodes, which were situated at either end of the muscle. Muscles were allowed to equilibrate for 15–20 min, during which time optimal length was determined and tetanic force stabilized. All contractions were displayed in an oscilloscope (Techtronix 2201) and then digitized on a chart recorder [1 kHz, 12-bit analog/digital (A/D), Keithley-MetraByte DAS1608] and stored on disk via microcomputer. Contractions were analyzed off-line for peak tetanic force (P₀). In the rested state, P₀ ranged from 20 to 25 g.

The assay buffer contained 250 mM sucrose, 20 mM HEPES (pH 7.5, 100 mM stock) and allowed to continue until little or no release was observed at 37°C. However, because of the lower coefficient of variation obtained at 37°C (see results), this temperature was used for all experiments.

Extravesicular free [Ca²⁺] was monitored fluorometrically using a Jasco CAF-110 Intracellular Ion Analyzer and indo 1 as the extravesicular Ca²⁺ indicator. Excitation light came from a high-pressure xenon lamp equipped with a monochromator and filtered at 349 nm. Emission fluorescence was determined by a pair of photomultipliers using 410 nm (F₄₁₀) and 500 nm (F₅₀₀) filters. Fluorescence ratios (R = F₅₀₀/F₄₁₀) were sampled at 2 Hz (Keithley-MetraByte DAS1608, 12-bit A/D) and stored on disk for later analysis. Free [Ca²⁺] was computed using the ratiometric method of Grynkiewicz et al. (13) using the following equation: [Ca²⁺]free = Kᵣ × β × (R - Rₘᵢₙ)/(Rₘₐₓ - Rₘᵢₙ), where the indo 1-Ca²⁺ dissociation constant (Kᵣ) was assumed to be 250 nM (13), Rₘᵢₙ and Rₘₐₓ are the R values measured in the uptake buffer with 10 mM EGTA added and with 1 mM Ca²⁺ added, respectively, and β is the ratio of F₅₀₀ recorded in EGTA- and Ca²⁺-supplemented buffers. The rates of Ca²⁺ uptake and release were computed from the steepest negative and positive slopes of the extravesicular free [Ca²⁺] versus time curve and normalized by the protein concentration. The initial fast phase of uptake, observed in Fig. 1 and reported by Gilchrist et al. (11), tended to be somewhat variable and was not considered in the calculation of uptake rate.

Statistical analyses. The effects of condition (rest, fatigue) and stimulation protocol on Ca²⁺ uptake and release were determined by repeated-measures analyses of variance adjusted for repeated measures made on contralateral muscles (split-plot). When needed, a Student-Newman-Keuls post hoc exam was used. Significance was set at the P < 0.05 level of confidence.

RESULTS

Validation of SR Ca²⁺ uptake and release measurement. A typical example of an SR Ca²⁺ uptake and release experiment is shown in Fig. 1. Our approach to measuring SR Ca²⁺ uptake and release using a homogenate fraction was found to be very reliable. Coefficients of variation for triplicate measurements made on an individual muscle were 3.56 ± 0.51 and 4.02 ± 0.49% (mean ± SE, n = 10) for uptake and release, respectively.
Table 1. Validation of SR Ca$^{2+}$ uptake and release methods

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Uptake</th>
<th>Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>11.32 ± 1.46</td>
<td>15.81 ± 2.14</td>
</tr>
<tr>
<td>CPA</td>
<td>8</td>
<td>0.07 ± 0.02*</td>
<td>ND</td>
</tr>
<tr>
<td>DTT</td>
<td>8</td>
<td>ND</td>
<td>0.10 ± 0.08*</td>
</tr>
<tr>
<td>NaN$_3$</td>
<td>6</td>
<td>12.17 ± 2.62</td>
<td>14.73 ± 3.82</td>
</tr>
</tbody>
</table>

Values are means ± SE. Rates are measured in nmol Ca$^{2+}$·mg sarcoplasmic reticulum (SR) protein$^{-1}$·min$^{-1}$. ND, not measured; CPA, cyclopiazonic acid; DTT, dithiothreitol; NaN$_3$, sodium azide. *P < 0.05 vs. control.

The technique of assessing SR Ca$^{2+}$ uptake and release used here relies on measurements of extravesicular Ca$^{2+}$ concentration. As opposed to measurements using isolated SR vesicles, the rates of extravesicular Ca$^{2+}$ change presented here could be influenced by Ca$^{2+}$ binding to other proteins and organelles present in the homogenate fraction. To ensure that our measurements accurately reflect SR Ca$^{2+}$ uptake and release, several initial experiments were performed (Table 1). First, we found that cyclopiazonic acid (20 µM), which inhibits the SR Ca$^{2+}$ ATPase (12), completely abolished Ca$^{2+}$ uptake. Second, AgNO$_3$, which is known to evoke SR Ca$^{2+}$ release via modification of specific sulfhydryl groups on the Ca$^{2+}$ release channel (18), initiated release in our samples. We found that dithiothreitol, which prevents the actions of AgNO$_3$ (18), completely inhibited Ca$^{2+}$ release. Finally, the inclusion of sodium azide (NaN$_3$) had no effect on either Ca$^{2+}$ uptake or release. Taken together, these results suggest that the rates of Ca$^{2+}$ uptake and release accurately reflect rates of SR Ca$^{2+}$ uptake and release rather than some nonspecific Ca$^{2+}$ binding and/or release.

Changes in SR Ca$^{2+}$ uptake and release. Changes in P$_0$ during the constant-fatigue experiments are shown in Fig. 2. There were no significant differences in the relative decline in P$_0$ between the three protocols. When data of all protocols were pooled, the average reduction in P$_0$ was 83% of initial. As anticipated, the time required for this reduction in force varied widely based on the protocol. The duration of activity was significantly longer (P < 0.05) at both 0.5 and 0.2 TPS than at 2.0 TPS. In addition, the number of stimuli required to reach the desired force output varied between protocols. Values at 0.2 TPS (202.8 ± 12.9) were significantly greater (P < 0.05) than those at 0.5 (130.5 ± 5.6) and 2.0 TPS (118.4 ± 5.7), which were not different from each other (P > 0.05). For the constant-duration experiments, stimulation was terminated after 1 min, which resulted in final forces of 15.3 ± 1.6, 55.3 ± 1.3, and 90.7 ± 2.3% of initial, respectively (P < 0.05 between all three conditions).

Changes in the rates of SR Ca$^{2+}$ uptake following fatigue during the constant-fatigue experiments are shown in Fig. 3. As can be seen, all three protocols resulted in significant reductions in uptake rate. Qualitatively similar results for the rates of Ca$^{2+}$ release are shown in Fig. 4. When either 25 µM AgNO$_3$ or 5 mM 4CMC was used, significant depressions in the Ca$^{2+}$ release rates occurred. It is important to point out that the amounts of Ca$^{2+}$ sequestered and released were not different between conditions (P > 0.05).

In the constant-duration experiments, the rate of Ca$^{2+}$ uptake was significantly reduced following the 2.0 and 0.5 TPS protocols only (Fig. 5). In addition, the magnitude of reduction following the 2.0 TPS protocol was greater than that which occurred following the 0.5 TPS protocol. Changes in the rates of AgNO$_3$- and 4CMC-induced release followed a similar pattern (Fig. 6). That is, they were reduced following 2.0 and 0.5 TPS stimulation only, with the depression being larger following the higher-frequency stimulation protocol (P < 0.05).

The difference in the Ca$^{2+}$ uptake and release rates between contralateral rested and fatigued muscles is shown in Fig. 7. As can be seen, the percent difference in rates during the constant-fatigue experiments was not significantly different between protocols. Each protocol resulted in a 40–50% reduction in Ca$^{2+}$ uptake and a similar reduction in Ca$^{2+}$ release induced by either AgNO$_3$ or 4CMC. On the other hand, the percent reductions in Ca$^{2+}$ uptake and release during the constant-duration experiments varied among each of the three protocols. The reductions in rates following stimulation at 0.5 TPS were approximately one-half of...
those that occurred following stimulation at 2.0 TPS, and the reductions in rates following 0.2 TPS were not different from zero.

DISCUSSION

The results of the constant-fatigue experiments show that when frog sartorius muscles are fatigued to similar extents, depressions in SR Ca\(^{2+}\) exchange kinetics are independent of the pattern of muscular activity. In each of the protocols used in the constant-fatigue experiments, an 83% reduction in Po resulted in 40–50% depressions in Ca\(^{2+}\) uptake and release rates, values that were not significantly different between protocols. These similar alterations occurred despite the fact that 1–17 min of stimulation were required to reach the desired reduction in Po. The results of the constant-duration experiments show that when muscles are stimulated for identical intervals, depressions in SR Ca\(^{2+}\) uptake and release rates vary considerably depending on the protocol used. With more intense activity patterns, where Po is reduced to a greater extent, larger depressions in SR function occur. With less intense activity, where little force depression is induced, SR function is not markedly altered.

It is important to point out that small reductions in force occurred without alterations in SR function. For example, stimulation at 0.2 TPS for 60 s resulted in a 10% reduction in Po without any reductions in Ca\(^{2+}\) uptake or release. Allen and colleagues (1, 7, 15, 19) show that during the initial minutes of stimulation, a small reduction in Po is associated with a rise in intracellular [Ca\(^{2+}\)] (\([\text{Ca}^{2+}]_i\)) during the tetanus, possibly reflecting enhanced SR Ca\(^{2+}\) release and/or depressed uptake. These authors suggest that the reductions in Po during “early fatigue” are likely the result of an altered intracellular milieu and the direct effects of elevated metabolite concentrations on force production by the contractile apparatus (1, 15, 19).

It is also important to recognize that Fitts et al. (10) found 26 and 74% reductions in rat soleus and extensor digitorum longus Po following 8 h of swimming. These

Fig. 4. Changes in rates of Ca\(^{2+}\) release in control (open bars) and fatigued (solid bars) muscles of the constant-fatigue experiments. A: release initiated by 25 µM AgNO\(_3\). B: release initiated by 5 mM 4-chloro-m-cresol (4CMC). *P < 0.05 vs. control.

Fig. 5. Changes in rates of Ca\(^{2+}\) uptake in control (open bars) and fatigued (solid bars) muscles of the constant-duration experiments. *P < 0.05 vs. control.

Fig. 6. Changes in rates of Ca\(^{2+}\) release in control (open bars) and fatigued (solid bars) muscles of the constant-duration experiments. A: release initiated by 25 µM AgNO\(_3\). B: release initiated by 5 mM 4CMC. *P < 0.05 vs. control.
changes in force, however, were not associated with alterations in the rates of SR Ca\textsuperscript{2+} uptake or in Ca\textsuperscript{2+} ATPase activities. On the other hand, several studies show that treadmill exercise lasting in excess of 1 h does result in depression in both Ca\textsuperscript{2+} uptake and release (6, 9, 16, 17). Unfortunately, muscle force output was not measured in these studies. It is possible that the mechanisms of force reduction during very prolonged activity such as that employed by Fitts et al. (10) do not involve changes in SR function. Perhaps some other mechanism such as glycogen depletion is operative.

The unique aspect of this investigation is the use of a single model for comparing SR Ca\textsuperscript{2+} uptake and release rates following different patterns of muscular activity. Others have shown that following many different types of activity, each resulting in fatigue or exhaustion, qualitatively similar depressions in the rates of Ca\textsuperscript{2+} uptake and release occur. Reductions in Ca\textsuperscript{2+} uptake and/or Ca\textsuperscript{2+} ATPase activity have been observed in humans, rodents, horses, and amphibians following both whole body exercise as well as electrical stimulation of isolated muscle (for review see Ref. 21). We found that changes in SR Ca\textsuperscript{2+} uptake and release rates were more dependent on the degree of fatigue rather than on the pattern of activity. In each protocol used, similar degrees of fatigue elicited similar reductions in the rates of Ca\textsuperscript{2+} uptake and release. Likewise, changes in Ca\textsuperscript{2+} uptake and release were observed only when stimulation elicited a noticeable reduction in P\textsubscript{0} (i.e., \(\geq 45\%\)). Thus it appears that changes in SR function are closely related to the reduction in tetanic force.

Similar conclusions have been reached by Allen et al. (1) and Baker et al. (2). They found a close relationship between P\textsubscript{0} and [Ca\textsuperscript{2+}], during both stimulation and recovery. Presumably, the changes in [Ca\textsuperscript{2+}], reflect altered SR Ca\textsuperscript{2+} release. This latter notion is supported by the present results and those of Williams and colleagues (20, 22), which show that changes in SR Ca\textsuperscript{2+} release are correlated with the degree of force loss. The primary difference between our results and those obtained using intact fibers (1, 2, 7, 15, 19) is that the changes in SR function shown here persist even though the SR was removed from its fatigued environment and examined in a medium whose composition more closely simulates that of a rested cell. Although the skinned fiber and homogenate fraction preparations used to assess SR function cannot fully reproduce conditions of an intact cell, our data are consistent with the idea that the relationship between P\textsubscript{0} and [Ca\textsuperscript{2+}], during fatigue (1, 2) results from reductions in the rates of SR Ca\textsuperscript{2+} uptake and release.

When viewed as a whole, our results and the previous work of others raise the possibility that intrinsic "dysfunctions" of the SR are important mechanisms of fatigue during activities in which noticeable reductions in force production occur. It appears that in a wide range of activities, fatigue (or exhaustion) appears to be associated with reductions in the kinetics of SR Ca\textsuperscript{2+} uptake and release. Such changes could directly cause the depression in P\textsubscript{0} that defines muscle fatigue. As muscular activity progresses, the diminished capability of the SR to adequately release and sequester Ca\textsuperscript{2+} results in decreased intracellular Ca\textsuperscript{2+} levels during contraction (1, 15, 19) and lowered activation of the contractile apparatus. As a consequence, P\textsubscript{0} is decreased.

Lukin et al. (16) argue that changes in SR Ca\textsuperscript{2+} uptake during fatigue are due to structural alterations in the Ca\textsuperscript{2+} ATPase that disrupt its catalytic cycle. In terms of Ca\textsuperscript{2+} release, Favero et al. (9) show that in addition to depressed AgNO\textsubscript{3}-induced release rates, ryanodine binding to its receptor is depressed following fatigue. Since ryanodine binds specifically to open Ca\textsuperscript{2+} release channels, this suggests that fewer channels respond to activating stimuli. In this investigation we used two agents, AgNO\textsubscript{3} and 4CMC, to induce SR Ca\textsuperscript{2+} release. AgNO\textsubscript{3} induces release by binding to thiols on the Ca\textsuperscript{2+} release channel (i.e., sulfhydryl oxidation) (18) and involves two mechanisms, one that is inhibited by ruthenium red and one that is not (5). On the other hand, 4CMC elicits Ca\textsuperscript{2+} release via direct interaction with the release channel (14, 23). That Ca\textsuperscript{2+} release rates induced by these agents were depressed to similar extents by fatigue suggests that fatigue reduces the number of release channels that respond to activating stimuli (e.g., AgNO\textsubscript{3} and 4CMC). Without doubt, more information is needed to establish a mechanism that

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**Fig. 7. Percent difference in Ca\textsuperscript{2+} uptake (open bars), AgNO\textsubscript{3}-stimulated release (hatched bars), and 4CMC-stimulated release (solid bars) between control and contralateral stimulated muscles.**

A: constant-fatigue experiments. B: constant-duration experiments.

*P < 0.05 vs. 2.0 and 0.2 TPS. †P < 0.05 vs. 2.0 and 0.5 TPS.

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accounts for the depressed Ca²⁺ release observed in fatigued muscle.

In summary, we show that despite differing stimulation protocols used to elicit fatigue in isolated muscle, similar reductions in P_o are associated with comparable reductions in SR Ca²⁺ uptake and release kinetics. In addition, SR function is only altered when P_o is markedly reduced. When coupled with previous work of others, these results suggest that SR dysfunction plays an important role in the fatigue process.

Perspectives

This investigation shows that during the development of fatigue, reductions in the rates of SR Ca²⁺ uptake and release are more closely associated with the reduction in P_o than with the pattern of activity. When these results are viewed in the context of studies that have shown that reductions in P_o during fatigue are associated with the decline in the tetanic Ca²⁺ transient (1, 15, 19), they emphasize the importance of altered excitation-contraction coupling in the fatigue process; specifically intrinsic changes in SR Ca²⁺ handling. As activity persists, reductions in SR Ca²⁺ transport likely result in depressed tetanic [Ca²⁺], reduced activation of the contractile apparatus, and diminished P_o. The alterations in SR Ca²⁺ uptake and release shown here probably do not result from the direct action of metabolites because the SR was removed from its "fatigued" environment and examined in one that more closely resembles that of a rested muscle. It is more likely that some change within the muscle triggers transient changes in SR structure and function that mediate the decline in Ca²⁺ handling (16). Potential candidates include the rise in resting [Ca²⁺], that accompanies fatigue (15), the production of reactive oxygen species, and/or the depletion of muscle glycogen. Each of these conditions has been shown to alter SR function in rested muscle (3, 4, 7, 20). For example, Chin and Allen (7) show that the loss of muscle glycogen is associated with a decline in tetanic [Ca²⁺]. Unfortunately, it remains to be seen which, if any, of these mechanisms triggers the depressions in SR Ca²⁺ transport that seemingly contribute to the development of fatigue.

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