Effects of ischemia on sarcoplasmic reticulum Ca\(^{2+}\) uptake and Ca\(^{2+}\) release in rat skeletal muscle

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The sarcoplasmic reticulum (SR) is a membranous structure in skeletal muscle that plays a critical role in the regulation of intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)). It acts both as a site of Ca\(^{2+}\) storage for subsequent release in response to muscle excitation and as a sink for removal of Ca\(^{2+}\) from the cytoplasm when excitation stops. In skeletal muscle, Ca\(^{2+}\) is stored primarily within the terminal cisternae of the SR, released through the 564-kDa Ca\(^{2+}\) release channel to initiate contraction, and resequestrered into the lumen of the SR by the 110-kDa SR Ca\(^{2+}\) ATPase pump, to allow relaxation (27).

In addition to its role in muscle contraction, it is well known that Ca\(^{2+}\) regulates numerous physiological cellular phenomena as a second messenger (45), as well as triggering pathological events such as cell injury and death (17). Therefore, a normally functioning SR is critical for normal cellular function and cellular survival.

In the heart, alterations in Ca\(^{2+}\) cycling with ischemia are well documented (30). It has been shown repeatedly that ischemia leads to a reduction in SR Ca\(^{2+}\) uptake, when measured in vitro (21, 22, 24, 28, 34, 35, 47, 49). Generally, reductions in Ca\(^{2+}\) uptake with myocardial ischemia are attributed to parallel reductions in Ca\(^{2+}\)-ATPase activity; however, there is some evidence to suggest that increased calcium release channel (CRC) activity and excessive Ca\(^{2+}\) leakage may also be involved (7, 12, 32).

It has also been shown that maximal Ca\(^{2+}\) release is depressed in vitro after myocardial ischemia in isolated rat hearts (26, 34). Several studies (19, 34, 48, 49) have also reported decreases in the number of function- ing CRCs, assessed by [\(^{3}\)H]ryanodine binding, in ischemic myocardium compared with control.

In contrast with the heart, very few studies have examined the effects of ischemia on SR Ca\(^{2+}\)-sequestering function in skeletal muscle, and, in particular, the effects of ischemia on SR Ca\(^{2+}\) uptake and SR Ca\(^{2+}\) release. Similar to the heart, skeletal muscle cells subjected to ischemia display a pronounced loss in function, which occurs relatively early in the ischemic period (4). However, unlike the heart, the maximal Ca\(^{2+}\)-ATPase activity in skeletal muscle, regardless of fiber type composition, has been reported to increase with prolonged ischemia (14). In addition, in contrast to the heart, where enriched SR fractions (24, 34) have been used to examine the ischemic effects on Ca\(^{2+}\)-ATPase activity, studies on skeletal muscle have employed a crude homogenate (14). Although the assay specificity for this preparation has been demonstrated (42), the possibility exists that other cellular ATPases could have been activated. Moreover, it has been re-

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ported (35, 47) that ischemia, at least in the heart, results in a differential yield of enriched SR, complicating the interpretation of the ischemic effects.

In this study, we employed a 4-h hindlimb ischemia model in rats to characterize the alterations in SR Ca\(^{2+}\) handling that occur with prolonged periods of skeletal muscle ischemia. In mixed fast-twitch muscle from ischemic and control hindlimbs, we measured both oxalate-supported Ca\(^{2+}\) uptake and silver nitrate (AgNO\(_3\))-induced Ca\(^{2+}\) release in both muscle homogenates and purified SR vesicles by use of the Ca\(^{2+}\) fluorescent dye Indo-1. We have hypothesized that, similar to the heart, ischemia would result in a reduction in both SR Ca\(^{2+}\) release and SR Ca\(^{2+}\) uptake, the magnitude of the effects dependent on the type of preparation.

METHODS

Animal Description and Care

Adult female Sprague-Dawley rats weighing 256 ± 6.7 g (means ± SE) were housed in an environmentally controlled room (temperature 22–24°C, 40–60% relative humidity) with reversed light-dark cycles. Animals were fed ad libitum on laboratory chow and water until the time of the experiment. All experiments were initiated at approximately the same time each day to avoid large diurnal variations in muscle glycogen (6). Experimental protocols were approved by the Animal Care Committee of the University of Waterloo.

Experimental Protocol

To investigate the effects of complete ischemia on SR Ca\(^{2+}\) uptake and Ca\(^{2+}\) release, animals were randomly assigned to control control (CC) (n = 10) and experimental (E) (n = 30) groups. For each E animal, the experimental condition of 4 h of total ischemia was randomly assigned to one hindlimb (I) while the contralateral limb served as a control limb (C). Because of tissue requirements for the isolation procedure used to obtain SR vesicles, experiments were conducted on one C and 3 E animals each day. Ischemia was induced by placing a tourniquet around the upper hindlimb and proximal to the knee joint. To ensure total occlusion of blood flow to the hindlimb, a 350-mmHg pressure was employed (44). Before the induction of ischemia, the rats were weighed and anesthetized. Anesthesia was initially accomplished with an intraperitoneal injection just under the skin before the induction of ischemia. To prevent dehydrated to 37 and 39°C by having the rats lie in a cage with reversed light-dark cycles. Animals were fed ad libitum on laboratory chow and water until the time of the experiment. All experiments were initiated at approximately the same time each day to avoid large diurnal variations in muscle glycogen (6). Experimental protocols were approved by the Animal Care Committee of the University of Waterloo.

Muscle metabolite analysis. To determine the effects of ischemia on muscle metabolic behavior, metabolites of the high-energy phosphate system (creatine phosphate (PCr), creatine (Cr), inorganic phosphate (Pi), glycogen (Glyc), and lactate (Lac)) were assessed. Metabolites were measured in freeze-dried tissue after perchloric acid extraction by fluorometric procedures, as previously reported (15). All concentrations were corrected for total Cr content (38).

Sample preparation for SR assessment in vitro. I and C muscles were prepared according to Heilmann et al. (18). Mixed GA and TA muscles were diluted to 1.5 wt/vol in ice-cold buffer containing (in mM) 5 HEPES (pH 7.5), 250 sucrose, 0.2% sodium azide, and 0.2 phenylmethylsulfonyl fluoride (no dithiothreitol) and mechanically homogenized with a polystyrene homogenizer (PT-310) at 1,500 rpm for 2–30 s bursts. Aliquots of the muscle homogenate were quick-frozen in liquid nitrogen and stored at −70 to −80°C for later analysis of SR function. The remainder of the homogenate was used to obtain an enriched SR membrane fraction through a combination of two SR isolation protocols (10, 18).

The homogenate was centrifuged at 5,500 g for 10 min to remove cellular debris, and the supernatant was filtered through four layers of gauze to remove as much fat as possible. The supernatant was then transferred to clean tubes and centrifuged at 12,500 g for 18 min. These pellets were discarded, and the spin was repeated. Again, the supernatant was transferred to clean tubes and centrifuged at 50,000 g for 52 min. These pellets were resuspended in 10 ml of homogenizing buffer plus 600 mM KCl and allowed to incubate at 4°C for 30 min. This suspension was then centrifuged at 15,000 g for 10 min to pellet nearly all the mitochondria. The supernatant obtained was centrifuged at 50,000 g for 52 min. The final pellet, enriched in SR membranes (no sucrose cushion), was resuspended in homogenizing buffer at a protein concentration of 2–6 mg/ml. SR isolation was carried out by differential centrifugation with a Beckmann Ultracentrifuge with a 70.1 Ti fixed-angle rotor.

SR Ca\(^{2+}\) uptake measurements. Oxalate-supported Ca\(^{2+}\) uptake was measured in muscle homogenates and purified SR by use of the Ca\(^{2+}\) fluorescent dye Indo-I, according to methods of O’Brien and colleagues (31, 33), with minor modifications. Fluorescence measurements were made on a spectrophotometer (RatioMaster system, Photon Technology International) equipped with dual emission monochromators. The measurement of [Ca\(^{2+}\)]\text{r} using the Indo-I procedure is based on the difference in the maximal emission wavelengths between the Ca\(^{2+}\)-bound form of Indo-I and the Ca\(^{2+}\)-free form. The excitation wavelength was 355 nm, and the emission maxima were 485 and 405 nm for Ca\(^{2+}\)-bound (F) and Ca\(^{2+}\)-free (G) Indo-I, respectively. Photon counts per second were recorded simultaneously for both emission wavelengths. The Ca\(^{2+}\)-independent (background) fluorescence was measured in the reaction medium (without Indo-I) at each emission wavelength before the experiment was started. Background fluorescence was automatically corrected with Felix software (Photon Technology International) before the start of each assay.

The reaction buffer for muscle homogenates (SR in parentheses) contained 200 (100) mM KCl, 20 mM HEPES, 10 mM NaNO\(_3\), 5 μM N,N,N',N'-tetrakis (2-pyridylmethyl)ethyl-enediamine (TPEN), 5 mM oxalate, 15 (10) mM MgCl\(_2\), and 10 μM phosphoenolpyruvate, pH 7.0. Before emission spectra were collected, 18 U/ml each of lactate dehydrogenase and pyruvate kinase and 1.5 μM of Indo-I were added to a cuvette containing 2 μl of reaction buffer. In addition, 3 μl of CaCl\(_2\)
(10 mM) were added at each trial to achieve an initial \([\text{Ca}^{2+}]_i\), before start of the reaction, of \(\sim 3.0 \mu \text{M}\). Immediately after data collection was initiated, 40\(\mu\)M of homogenate (20\(\mu\)L of SR) were added to the cuvette. Shortly after the addition of homogenate or SR, 5 mM ATP was added to initiate Ca\(^{2+}\) uptake.

As Ca\(^{2+}\) decreases because of active SR Ca\(^{2+}\) uptake, F decreases, G increases, and the ratio (R) of F to G, which is used to calculate \([\text{Ca}^{2+}]_i\), decreases. The ratio (R) is used to calculate \([\text{Ca}^{2+}]_i\). With Felix software, the ionized Ca\(^{2+}\) concentration was calculated by the following equation (16):

\[
[\text{Ca}^{2+}]_i = K_d \cdot \frac{(G_{\text{max}}/G_{\text{min}})(R - R_{\text{min}})/(R_{\text{max}} - R)}{}
\]

where \(K_d\) is the equilibrium constant for the interaction between Ca\(^{2+}\) and Indo-I, \(R_{\text{min}}\) is the minimum value of R at addition of 250 \(\mu\)M EGTA, \(G_{\text{max}}\) is the maximum value of G at addition of 250 \(\mu\)M EGTA, \(G_{\text{min}}\) is the minimum value of G at addition of 1 mM CaCl\(_2\), and \(R_{\text{max}}\) is the maximum value of R at addition of 1 mM CaCl\(_2\). The \(K_d\) value for the Ca\(^{2+}\) dye complex is 250 and 135 nM for muscle homogenates and purified SR vesicles, respectively (16). For all Ca\(^{2+}\) uptake trials, \(R_{\text{min}}\) and \(R_{\text{max}}\) were not determined until Ca\(^{2+}\) uptake had plateaued, which occurred at \(\sim 100 \text{nM}[\text{Ca}^{2+}]_i\).

Before the rate of Ca\(^{2+}\) uptake was calculated, the generated curve from Eq. 1, \([\text{Ca}^{2+}]_i\) vs. time, was smoothed over 21 points using the Savitsky-Golay algorithm. The rate of Ca\(^{2+}\) uptake was then analyzed at four separate free calcium concentrations (0.5, 1.0, 1.5, and 2.0 \(\mu\)M) for both muscle homogenates and SR. First, linear regression was done on a range of values 100 nM above and below the desired \([\text{Ca}^{2+}]_i\).

The rate of Ca\(^{2+}\) uptake was then determined by differentiating the linear-fit curve and expressing the values in micromoles per gram of protein per minute.

In a subset of homogenate samples (\(n = 5\)), Ca\(^{2+}\) uptake assays were run with and without 300 \(\mu\)M ryanodine to assess the possible role of the CRC channel in altering Ca\(^{2+}\) uptake with ischemia. There is some evidence to suggest that increased CRC activity and excessive Ca\(^{2+}\) leakage at rest may contribute to an increase in \([\text{Ca}^{2+}]_i\), producing erroneous values for Ca\(^{2+}\) uptake with ischemia (7, 32).

SR Ca\(^{2+}\) release measurements. Ca\(^{2+}\) release was measured on both muscle homogenates and SR vesicles according to the methods of Ruell et al. (37), with minor modifications. Ca\(^{2+}\) release assays were conducted as for Ca\(^{2+}\) uptake, except that when the \([\text{Ca}^{2+}]_i\) declined to a plateau, 3 \(\mu\)L of AgNO\(_3\) were added to give a final concentration of 141 \(\mu\)M AgNO\(_3\). The reaction was then allowed to proceed for \(\sim 3\) min. In muscle homogenates only, we also measured Ca\(^{2+}\) release in the presence of 40 \(\mu\)M cyclopiazonic acid (CPA), which completely inhibits SR Ca\(^{2+}\)-ATPase activity and Ca\(^{2+}\) uptake (40). In the trials with CPA, 40 \(\mu\)M CPA were added after active loading of the SR with Ca\(^{2+}\) and just before the addition of AgNO\(_3\). This was done to assess the effects of ischemia on Ca\(^{2+}\) release independent of the changes in Ca\(^{2+}\) uptake that may occur with ischemia. In a subset of muscle homogenate samples (\(n = 5\)), in the presence and absence of CPA, Ca\(^{2+}\) release was allowed to proceed until a plateau was reached to measure total Ca\(^{2+}\) release in each group. This procedure was intended to assess the amount of loading of calcium in the SR before the initiation of release.

With the addition of AgNO\(_3\), Ca\(^{2+}\) release consistently proceeded in two distinct phases. There was an initial rapid rate of release (phase 1) followed by a slower, more prolonged rate of release (phase 2) (Fig. 1). As with Ca\(^{2+}\) uptake, the generated curve from Eq. 1 was smoothed over 21 points and differentiated. The maximal rate of Ca\(^{2+}\) release was calculated by taking the maximum positive derivative for each phase and expressing values in millimoles per gram of protein per minute. For both Ca\(^{2+}\) uptake and Ca\(^{2+}\) release, protein was determined by the method of Lowry, as modified by Schacterle and Pollock (39). On a given day, equal numbers of samples from each condition and for a given variable were analyzed in duplicate.

Muscle force measurements. In a separate group of ischemic animals (\(n = 5\)), the effects of I on electronically evoked muscle twitch force were assessed for the gastrocnemius-plantaris-soleus complex from each hindlimb. Surgical preparation and the animal stimulation apparatus have been described in detail elsewhere (4, 5). For all animals, peak twitch force (\(P_t\)) was measured at rest and at 15, 30, 45, 60, 120, 180, and 240 min of ischemia. Twitch force was obtained via direct muscle stimulation using stainless steel electrodes, with a single 0.2-ms pulse at 70 V. Muscle stimulation was performed using a Gras S48 Stimulator while force data were collected on line and analyzed using the Watscope Data Acquisition Unit and Software (Northern Digital 1985–1987). An independent shunt calibration was employed to avoid problems associated with mechanical calibration. Before induction of ischemia, optimal length (\(L_o\)) for maximal \(P_t\), and maximal voltage for optimum muscle twitch force were established. In determining \(L_o\), muscle twitches were separated by 30 s to avoid a fatigue or potentiation effect. After \(L_o\) and supramaximal voltage were established, the muscle was allowed to equilibrate for 5 min before ischemia was induced.

Twitch force was expressed relative to the initial resting value and calculated as a percentage of initial force.
Data Analysis

For Ca\(^{2+}\) release measurements in muscle homogenates, a two-way ANOVA was used to discriminate between differences due to assay (with vs. without CPA) and group (CC vs. C vs. I). Where an overall interaction between assay and group was found, post hoc analyses (Tukey’s) were performed to determine specific assay and group effects. For all other measurements, a one-way ANOVA was used to test for differences between means. Where significant differences were found, Tukey’s post hoc tests were used to compare specific means. For all comparisons, statistical significance was accepted at \(P < 0.05\). All data are expressed as means ± SE.

RESULTS

Force Measurements

The changes in Pt with I and C are presented in Fig. 2. By 2 h of ischemia, the muscle was incapable of producing force and remained in this state for the duration of the ischemic protocol. Importantly, Pt force output was maintained in C throughout the I protocol.

Muscle Metabolites

Muscle metabolites for each group are shown in Table 1. With the exception of Pi, there were no differences between CC and C for any of the metabolites measured. Muscle ATP and PCr concentrations were almost fully depleted in I, both reaching levels below 2 mmol/kg dry wt. Compared with CC and C, muscle Cr and P, were ~82 and 350% higher \((P < 0.05)\) in I, respectively. Lac was 12-fold higher \((P < 0.05)\) and Glyc was 88% lower \((P < 0.05)\) in I compared with CC and C.

\[Ca^{2+}\] Uptake

\[Ca^{2+}\] uptake was assessed at four different values of \([Ca^{2+}]_f\) in both muscle homogenates and purified SR vesicles. In muscle homogenates, \[Ca^{2+}\] uptake was 25–28% lower \((P < 0.05)\) in I, compared with CC and C, across all \([Ca^{2+}]_f\) values (Fig. 3). The addition of 300 \(\mu\)M ryanodine in the homogenate \[Ca^{2+}\] uptake buffer \((n = 5)\), to inhibit \[Ca^{2+}\] release channel opening, had no effect on \[Ca^{2+}\] uptake in any of the groups at either 500 or 1,000 nM \([Ca^{2+}]_f\) (Fig. 4). However, a main effect \((P < 0.05)\) of ryanodine was observed at 500 nM such that \[Ca^{2+}\] uptake was generally lower across conditions in the presence of ryanodine compared with no ryanodine. In purified SR vesicles, \[Ca^{2+}\] uptake was also lower \((P < 0.05)\) in I by 40–46% across all values of \([Ca^{2+}]_f\) compared with CC and C (Fig. 5). There were no differences between CC and C in either muscle homogenates or purified SR vesicles.

![Fig. 2. Changes in peak twitch tension (Pt) during ischemia of C and I for the gastrocnemius-plantaris-soleus complex. Values are means ± SE; n = 5 per group.](image)

Table 1. Muscle metabolite concentrations for ischemic and control groups

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>C</th>
<th>I</th>
</tr>
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<tbody>
<tr>
<td>ATP</td>
<td>28.3 ± 0.5</td>
<td>27.5 ± 0.3</td>
<td>1.1 ± 0.3*</td>
</tr>
<tr>
<td>PCr</td>
<td>77.1 ± 2.1</td>
<td>77.2 ± 3.0</td>
<td>1.3 ± 0.4*</td>
</tr>
<tr>
<td>Cr</td>
<td>84.9 ± 2.0</td>
<td>78.5 ± 2.5</td>
<td>150.0 ± 2.3*</td>
</tr>
<tr>
<td>Pi</td>
<td>46.8 ± 1.5</td>
<td>37.6 ± 2.0†</td>
<td>163.7 ± 3.4*</td>
</tr>
<tr>
<td>Lac</td>
<td>22.4 ± 1.0</td>
<td>24.5 ± 2.7</td>
<td>249.0 ± 5.6*</td>
</tr>
<tr>
<td>Glyc</td>
<td>117.3 ± 2.6</td>
<td>118.8 ± 3.7</td>
<td>14.1 ± 1.0*</td>
</tr>
</tbody>
</table>

Values are means ± SE in \(\mu\)mol/g dry wt with the exception of glycogen (Glyc), which is in \(\mu\)mol glucosyl units/g dry wt \((n = 10\) per group). CC, control control; C, control (limb); I, ischemic; PCr, phosphocreatine; Cr, creatine; Pi, inorganic phosphate; Lac, lactate. *Significantly different \((P < 0.05)\) from CC and C. †Significantly different \((P < 0.05)\) from CC.

![Fig. 3. Calcium uptake of CC, C, and I homogenates prepared from mixed gastrocnemius and tibialis anterior muscles. Ca\(^{2+}\) uptake was measured using Indo-I and at 4 submaximal \([Ca^{2+}]_f\) values (2,000, 1,500, 1,000, and 500 nM). Values are means ± SE; n = 9. *Significantly different from CC and C.](image)
Because of the biphasic response of Ca\textsuperscript{2+} release, we obtained two measures of maximal Ca\textsuperscript{2+} release, corresponding to the two phases, for each assay (Fig. 1). In muscle homogenates, both with and without CPA, maximal Ca\textsuperscript{2+} release for phase 1 was not different between groups (Fig. 6). For phase 1, maximal release was higher (\(P < 0.05\)) in the presence of CPA than without CPA in all groups. On the other hand, for phase 2, in the absence of CPA, maximal Ca\textsuperscript{2+} release was 24% lower (\(P < 0.05\)) in I compared with CC and C.

### Ca\textsuperscript{2+} Release

However, there were no differences between groups for phase 2, when Ca\textsuperscript{2+} release was measured in the presence of CPA. Unlike phase 1, maximal Ca\textsuperscript{2+} release for phase 2 was lower (\(P < 0.05\)) in all groups when measured in the presence of CPA compared with no CPA. There were no differences between CC and C in any condition. In purified SR vesicles, maximal Ca\textsuperscript{2+} release was 26 and 54% lower (\(P < 0.05\)) in I, compared with CC and C, for phase 1 and phase 2, respectively (Fig. 7). No differences existed between CC and C for either phase. Total Ca\textsuperscript{2+} release was assessed in a subset of muscle homogenate samples (\(n = 5\)) to ensure...
that SR Ca\(^{2+}\) loading was similar in all groups, before the initiation of Ca\(^{2+}\) release. Total Ca\(^{2+}\) release represents the total amount of Ca\(^{2+}\) that was released from the SR until a plateau was reached and release of Ca\(^{2+}\) stopped. For the no CPA condition, the values were 2,196 ± 165, 2,386 ± 209, and 2,283 ± 209 \(\mu M\) for the CC, C, and I groups, respectively. For the CPA condition, comparable values were 1,846 ± 315 (CC), 1,705 ± 112 (C), and 1,781 ± 297 (I) \(\mu M\). There were no differences in total Ca\(^{2+}\) release among groups, regardless of whether CPA was present or not (data not shown). However, there was a main effect (\(P < 0.05\)) for CPA, where total release was lower with CPA than without CPA.

**DISCUSSION**

In the present study, in vitro measures of both SR Ca\(^{2+}\) uptake and Ca\(^{2+}\) release were made on both SR vesicles and muscle homogenates prepared from rat skeletal muscle tissue that was made ischemic in vivo for 4 h. As expected, we found evidence to support our hypothesis, namely, that both SR Ca\(^{2+}\) uptake and Ca\(^{2+}\) release are impaired with prolonged skeletal muscle ischemia. Moreover, at least for Ca\(^{2+}\) uptake, the effect of ischemia was the same in both homogenates and SR vesicles. However, for Ca\(^{2+}\) release, the effects of ischemia, although present in both phases in the SR vesicles, were found only in phase 2 of the homogenates. This suggests that SR dysfunction plays a role in the observed calcium overload that occurs with skeletal muscle ischemia (36). The changes in SR function were also accompanied by a nearly 100% loss in force-generating capabilities and by an almost total depletion of ATP and PCr. Both force and energy charge in the contralateral control (C) muscles of the ischemic animals and in the muscles sampled immediately after anesthesia were unaltered.

**Ca\(^{2+}\) Uptake**

The rate of Ca\(^{2+}\) uptake in this study was assessed across a range of submaximal [Ca\(^{2+}\)]\(_{i}\) values. The reduction in Ca\(^{2+}\) uptake with ischemia was consistent across all [Ca\(^{2+}\)]\(_{i}\) values, regardless of whether it was measured in muscle homogenates or SR vesicles. Unfortunately, we were unable to determine maximal Ca\(^{2+}\) uptake rates in either homogenates or SR vesicles with any confidence; therefore, calculation of the Hill coefficient and the [Ca\(^{2+}\)]\(_{i}\) at half-maximal activity was not possible. Maximal Ca\(^{2+}\)-ATPase activity in these samples occurs at a [Ca\(^{2+}\)]\(_{i}\) of ~6–10 \(\mu M\) (unpublished observation). Because the highest [Ca\(^{2+}\)]\(_{i}\) used to measure the rate of Ca\(^{2+}\) uptake in this study was 2.0 \(\mu M\), it is unlikely that maximal Ca\(^{2+}\) uptake rates occurred at this level.

The net rate of Ca\(^{2+}\) uptake depends on two processes, namely, Ca\(^{2+}\) accumulation by the SR and Ca\(^{2+}\) efflux from the SR. Ca\(^{2+}\) accumulation is controlled by the activity of the SR Ca\(^{2+}\)-ATPase pump. Therefore, reductions in Ca\(^{2+}\) uptake with ischemia could be due to reductions in Ca\(^{2+}\)-ATPase activity or an increased efflux of Ca\(^{2+}\) from the SR. An increased efflux of Ca\(^{2+}\) may be due to either an increased permeability of the SR membrane to Ca\(^{2+}\) or an increase in the passive release of Ca\(^{2+}\) through CRC (35). In the current study, the reduction in Ca\(^{2+}\) uptake with prolonged ischemia does not appear to be due to excessive Ca\(^{2+}\) leakage through the CRC, as Ca\(^{2+}\) uptake was reduced to the same extent in ischemic homogenate samples when measured either with or without ryanodine. We can safely assume that most of the reduction in Ca\(^{2+}\) uptake with ischemia in this study can be explained by impaired SR Ca\(^{2+}\) pump function. In fact, we have observed that maximal activity of the Ca\(^{2+}\) pump is reduced after 4 h of ischemia in rat skeletal muscle (43).

**Ca\(^{2+}\) Release**

This study appears to be the first to assess the effects of ischemia on SR Ca\(^{2+}\) release in vitro, in skeletal muscle. One study in the heart has reported that EGTA-induced Ca\(^{2+}\) release, measured in SR vesicles prepared from the left ventricle, was reduced after 30 min of global ischemia (34). In our study, we also found reductions in maximal Ca\(^{2+}\) release with prolonged ischemia, measured in SR vesicles. The Ca\(^{2+}\) release technique employed in this study, AgNO\(_3\)-induced Ca\(^{2+}\) release, resulted in a Ca\(^{2+}\) release response that was biphasic in nature. In SR vesicles, the peak rate of Ca\(^{2+}\) release corresponding to each phase was lower in ischemic SR vesicles compared with control. The mechanism for this biphasic response is unknown; however, the clear distinction between the two phases, as can be seen in Fig. 1, and the differential effects of CPA on the two phases indicate the value of reporting data on each phase.

The peak rate of Ca\(^{2+}\) release measured in homogenates, unlike in SR vesicles, was only lower in phase 2 and not in phase 1. The different results between SR vesicles and homogenates may be due to differential selection of a population of SR vesicles, between ischemic and control muscle, during the isolation procedure, as has been reported in the heart after ischemia and reperfusion (35, 46). On the other hand, the rapid release rate characteristic of phase 1, as measured by Indo-1, may be affected to a greater extent by the various cytosolic Ca\(^{2+}\)-binding proteins than phase 2, and this would certainly be different between SR vesicles and homogenates. Because the effects of ischemia on SR Ca\(^{2+}\) uptake were similar between SR vesicles and homogenates, we suggest that the reduction in the peak rate of Ca\(^{2+}\) release corresponding to phase 1 in SR vesicles was likely due to ischemia and not to the isolation procedure itself.

In this study, we also measured AgNO\(_3\)-induced Ca\(^{2+}\) release in the presence of CPA, to block Ca\(^{2+}\) uptake during net Ca\(^{2+}\) release in homogenates only. Given that the net Ca\(^{2+}\) release by the SR is a result of the opposite activities of the CRC and the Ca\(^{2+}\)-ATPase, an abnormal Ca\(^{2+}\) release may be caused by a dysfunction of either or both structures. Therefore,
CPA was used to assess the effects of ischemia on SR Ca\(^{2+}\) release function independent of changes in Ca\(^{2+}\) uptake. For this technique to be effective, the timing of CPA addition is critical. It has been shown in both actively loaded SR vesicles (8) and skinned skeletal muscle fibers (9) that CPA inhibition of the SR Ca\(^{2+}\) pump induces a slow release of Ca\(^{2+}\) from the SR. Given the influence of both external and luminal Ca\(^{2+}\) on SR Ca\(^{2+}\) release, interpretation of the effects of CPA on Ca\(^{2+}\) release depends on the length of the incubation period of SR vesicles with CPA. Because Ca\(^{2+}\) release was initiated by the addition of AgNO\(_3\) immediately after addition of CPA, consequently minimizing possible CPA-induced Ca\(^{2+}\) leakage from the SR, it would appear that the effects of CPA on Ca\(^{2+}\) release were directly related to inhibition of Ca\(^{2+}\) pump activity and Ca\(^{2+}\) uptake, and not to altered Ca\(^{2+}\) levels on either side of the SR membrane before release.

We found that Ca\(^{2+}\) release rates for phase 1 were higher in the presence of CPA for all groups, with no differences among groups. However, for phase 2, Ca\(^{2+}\) release rates were lower with CPA across all groups. If the rate of Ca\(^{2+}\) release simply reflects a balance between the rate of release and reuptake of Ca\(^{2+}\) during release, then this finding is opposite to what would have been expected. This finding suggests that some Ca\(^{2+}\) pump activity and Ca\(^{2+}\) uptake during net Ca\(^{2+}\) release are necessary for a normal release response. In accord with this interpretation, the reduction in Ca\(^{2+}\) uptake with ischemia may contribute to the reduction in Ca\(^{2+}\) release that occurs with ischemia, at least for phase 2.

Although we did not measure the number of functional CRC, as assessed by a decrease in \([^{3}H]}\)ryanodine binding capacity in SR vesicles, it would be expected that a decrease would occur with prolonged ischemia. This finding has been reported in several studies (20, 34, 48) with myocardial ischemia and reperfusion. It is possible that most of the reduction in peak Ca\(^{2+}\) release for phase 1 and phase 2 in SR vesicles can be explained by a reduced number of functioning CRC with ischemia.

Damage to the CRC and reductions in Ca\(^{2+}\) release function with ischemia may in fact be secondary to reductions in Ca\(^{2+}\) uptake and elevations in [Ca\(^{2+}\)]\(_i\), as suggested during repetitive activity (11). Calcium may indirectly lead to protein damage from its activation of the neutral protease calpain (2). Ca\(^{2+}\)-activated calpain-induced proteolysis of the CRC has been demonstrated in heavy SR membranes; however, proteolysis did not alter the Ca\(^{2+}\)-handling or ryanodine-binding properties of the heavy SR membranes (13). This would suggest that other mechanisms may also be involved with ischemia to explain the reduction in Ca\(^{2+}\) release that occurs. One possibility is O\(_2\) free radicals. It is well established that free radical formation plays a primary role in the etiology of ischemia-induced damage in skeletal muscle (36). Very few studies have reported the effects of free radicals on SR CRC (23). It is apparent, however, that free radicals can trigger rapid Ca\(^{2+}\) release from the SR by modifying critical sulfhydryls in the CRC and increasing the open probability of the channel (for review see Ref. 1). If this occurs with ischemia, elevations in Ca\(^{2+}\) release through the Ca\(^{2+}\) release channel at rest may contribute to the rise in [Ca\(^{2+}\)]\(_i\) and Ca\(^{2+}\) overload that occurs with ischemia. However, we found that Ca\(^{2+}\) release was reduced with ischemia. In agreement with this, Brotto and Nosek (3) found that hydrogen peroxide inhibited Ca\(^{2+}\) release in skinned skeletal muscle fibers from the rat. It is also possible that an initial increase in the probability of the channel being in the open state is followed by irreversible loss of channel function as a result of free radical damage (19).

The reduction in Ca\(^{2+}\) uptake with ischemia is probably related to free radical-induced damage to the Ca\(^{2+}\)-ATPase. The Ca\(^{2+}\)-ATPase contains 24 cysteine residues and, consequently, the SR Ca\(^{2+}\)-ATPase may be a principal target for modulation of muscle function by reactive oxygen species (25), as has been demonstrated in numerous in vitro studies (29, 41).

In this study, it is important to remember that we employed an in vivo model of skeletal muscle ischemia and that SR transport function was assessed in vitro under ideal conditions. Assuming assay validity, any ischemia-induced changes in Ca\(^{2+}\) uptake or Ca\(^{2+}\) release measured in vitro must be due to intrinsic changes such as structural alterations in either one or more of the SR proteins, the phospholipid membrane of the SR, or both. Although our in vitro observations of a reduced Ca\(^{2+}\) uptake and Ca\(^{2+}\) release are important, reductions in both would be even greater in vivo because of the additional local metabolic effects (48). No measures of membrane integrity were performed in this study.

The validity of using “in vitro” measures to determine SR Ca\(^{2+}\) uptake and Ca\(^{2+}\) release with the fluorescent dye Indo-1 is based on several conditions, including minimizing the role of mitochondrial and sarcoplasmal Ca\(^{2+}\) exchange and passive binding and unbinding to proteins and phospholipids. In addition, it also appears that changes in the Ca\(^{2+}\)-binding ligand Indo-I, as might occur during tissue uptake of Indo-I, have a negligible effect on Ca\(^{2+}\) cycling measurements. All of these issues have been addressed previously (32).

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