Skeletal muscle sarcoplasmic reticulum glycogen status influences Ca$^{2+}$ uptake supported by endogenously synthesized ATP

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Lees, Simon J., and Jay H. Williams. Skeletal muscle sarcoplasmic reticulum glycogen status influences Ca$^{2+}$ uptake supported by endogenously synthesized ATP. Am J Physiol Cell Physiol 286: C97–C104, 2004—The purpose of this investigation was to determine whether there is a link between sarcoplasmic reticulum (SR) glycogen status and SR Ca$^{2+}$ handling. In this investigation, skeletal muscle SR was purified from female Sprague-Dawley rats (200–250 g). Glycogen was extracted from the SR purified from one hindlimb, whereas the SR purified from the contralateral limb served as control. Before removal of the tissue, the animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Both α-amylase treatment (AM) and removal of EDTA from the homogenization and storage buffers reduced the amount of glycogen associated with the SR (P < 0.05). AM treatment reduced the glycogen phosphorylase content of SR (P < 0.05). In contrast, creatine kinase (CK) and pyruvate kinase (PK) contents were increased after both glycogen extraction protocols (P < 0.05). Under exogenous ATP conditions, both AM and EDTA-free (EF) treatments resulted in an increase in Ca$^{2+}$-stimulated ATPase activity when normalized to sarco(endo)plasmatic reticulum calcium-ATPase (SERCA) content (P < 0.05). CK and PK-supported SR Ca$^{2+}$ uptake was decreased (P < 0.05) in the AM group when normalized to SERCA and CK or SERCA and PK content, respectively. AM was more effective than the EF for extracting glycogen associated with purified SR. Glycogen extraction alters the yield of purified SR proteins and must be taken into account when investigating SR calcium handling. Removal of glycogen from purified SR causes a change in Ca$^{2+}$-handling properties as measured by ATPase and uptake activities.

glycogen extraction; fatigue; SERCA

Prolonged muscular exercise typically results in marked reductions in the level of muscle glycogen. Over the past 30 years, a close relationship between muscle glycogen and exercise exhaustion has emerged. For example, the rate of glycogen depletion in exercising muscle is dependent on the intensity of the exercise (35), the time to exhaustion during endurance exercise is linked to the level of pre-exercise muscle glycogen (5), and carbohydrate ingestion during exercise increases time to fatigue (7, 9) as well as sprint performance after prolonged exercise (17).

Prolonged exercise is also accompanied by alterations in intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) handling (for reviews see Refs. 39, 41). Specifically, several groups report that the rates of Ca$^{2+}$ uptake and release by the sarcoplasmic reticulum (SR) are depressed after exercise and repetitive stimulation (6, 16, 40, 42). These changes are not a result of accumulated metabolites, because diminished SR Ca$^{2+}$ handling is observed even when the SR has been removed from its “fatigued” environment and examined in one that more closely mimics that of a rested cell. Thus fatigue seems to induce some intrinsic alteration in SR function.

Certainly, there is ample evidence that skeletal muscle fatigue is accompanied by changes in the sarco(endo)plasmatic reticulum Ca$^{2+}$-ATPase (SERCA) function itself (e.g., depressed SR Ca$^{2+}$ uptake and Ca$^{2+}$-stimulated ATPase activity in the presence of excess exogenous ATP). Furthermore, Luckin et al. (29) found altered SERCA conformation states, as measured by fluorescein isothiocyanate (FITC) binding, in SR purified from fatigued muscle and suggested that this might be related to depressed SERCA function. Even though SR dysfunction is still evident when it is removed from the intracellular milieu, it is important to consider physiologically relevant metabolic processes that are integral for optimal in vivo function. Glycogenolytic and glycolytic enzymes, as well as creatine kinase (CK), are associated with the SR membrane (2, 15, 22, 23, 24, 36, 45, 46). Xu et al. (45) that SERCA seems to be located at adjacent positions on SR vesicles with pyruvate kinase (PK), aldolase, and glyceralddehyde-phosphate dehydrogenase. Furthermore, these metabolic systems are needed for optimal SR Ca$^{2+}$ uptake (13, 43) and in some cases are more efficient than exogenous ATP (46). Despite the obvious significance of these metabolic systems in vivo, very little is known about their function in either a fatigued muscle or when glycogen status is depressed.

Recently, we have shown that the level of SR glycogen is reduced after repetitive stimulation of rat gastrocnemius muscle. In addition, the content of glycogen phosphorylase is reduced (26). It is possible that the reduction in SR glycogen and SR Ca$^{2+}$ handling are causally linked. Mechanically skinned muscle fibers respond better to T-system depolarization under conditions in which the glycogen content is either preserved (38) or elevated by incubation with insulin and glucose (1). Chin and Allen (10) show that when mouse fibers are stimulated to fatigue and then allowed to recover in a glucose-free solution, [Ca$^{2+}$]$_i$ handling remained depressed and a subsequent fatigue bout resulted in a greater and more rapid force loss. Korge and Campbell (23) found that SR purified from fatigued muscle had reduced SR Ca$^{2+}$ uptake supported by either exogenous ATP or endogenously synthesized ATP via CK. More importantly, these investigators reported that in SR purified from fatigued muscles, endogenous CK-supported SR Ca$^{2+}$ uptake was depressed to a greater extent than that supported by exogenous ATP (46).
extent than exogenous ATP-supported SR Ca\(^{2+}\) uptake. However, these results of Chin and Allen (10) and Korge and Campbell (23) do not indicate whether the changes in Ca\(^{2+}\) handling were due specifically to the loss of glycogen or to some other factor. In 2002, Batts (3) measured in situ muscle function in glycogen-depleted rats. This investigator reported that depressed SR glycogen was associated with decreased relaxation rates, which is consistent with decreased SR Ca\(^{2+}\) uptake rates in the intact cell.

The cellular localization of CK and glycogenolytic and glycolytic enzymes to the SR, combined with the fact that these systems are able to support SR Ca\(^{2+}\) uptake, indicates their physiological importance. Furthermore, endogenous CK-supported SR Ca\(^{2+}\) uptake is diminished in SR purified from fatigued muscle (23). However, there has been no published research on the effects of glycogen extraction on glycolytic and CK-supported SR Ca\(^{2+}\) uptake. In this investigation, we extracted glycogen from isolated SR vesicles to determine whether there is a causal link between glycogen status of the SR and SR Ca\(^{2+}\) handling. Here, we show that extracting glycogen from SR vesicles results in a slight stimulation of Ca\(^{2+}\) uptake, Ca\(^{2+}\)-stimulated ATPase activity, and Ca\(^{2+}\) release rates in vitro in the presence of exogenous ATP. However, SR Ca\(^{2+}\) uptake rates were decreased in α-amylase-treated samples when supported by endogenous ATP synthesis.

**METHODS**

**Animals.** The Virginia Tech Institutional Animal Use and Care Committee approved all procedures for the present investigation. Female Sprague-Dawley rats were used in this investigation (200–250 g, n = 6 in each group). Animals were housed two per cage and allowed free access to standard rodent chow and water. Before death, they were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg).

**Tissue processing.** The gastrocnemius-plantaris muscle group was harvested from each leg and designated as a control and either AM or EF group. The muscle was placed in ice-cold homogenization buffer containing 20 mM HEPES, 0.2% sodium azide, 0.2% PMSF, and 1 mM EDTA (pH 7.0). The AM group homogenizing buffer was supplemented with 0.1% α-amylase (EC 3.2.1.1) during the KCl incubation, and the EF group homogenization buffer was EDTA free. The muscle was then minced and homogenized for three 15-s bursts at 10,000 rpm (Virtishear). Homogenates were centrifuged at 8,000 g for 15 min at 4°C. The supernatant was filtered through four layers of gauze, and KCl was added to a final concentration of 600 mM KCl. Samples were incubated on ice, with gentle shaking, for 1 h. Supernatants were then centrifuged at 43,000 g for 120 min. The resulting pellet (purified SR vesicles) was resuspended in storage buffer (homogenizing buffer containing 300 mM sucrose and 150 mM KCl). For vesicles used in SR glycogen measurements, the storage buffer did not contain sucrose because it interferes with that assay.

**Biochemical measurements.** SR glycogen was determined using the enzymatic method determined by the Keppeler and Deck (20) and modified by Lees et al. (26). Briefly, glycogen was hydrolyzed by glucoamylase (EC 3.2.1.3), and glucose was then measured fluorometrically using the production of NADPH.

**SR Ca\(^{2+}\) uptake.** SR vesicles (25 µg of SR protein) were placed in a buffer containing 100 mM KCl, 20 mM HEPES, 1 mM MgCl\(_2\), 5 mM KH\(_2\)PO\(_4\), 2 mM ATP, and 250 µM antipyrylazo III (APIII) (pH 7.0, 37°C). For CK-supported uptake experiments, 2 mM ADP and 5 mM creatine phosphate (CP) were included in the uptake buffer in place of the 2 mM ATP. For PK-supported uptake experiments, 2 mM ADP and 5 mM of phosphoenol-pyruvate (PEP) were included in the uptake buffer in place of the 2 mM ATP. Uptake was initiated by adding 1.2 µmol/mg CaCl\(_2\) and was allowed to continue until free Ca\(^{2+}\) in the cuvette declined to a plateau. A diode array spectrophotometer (Hewlett Packard 8453) was used to follow APIII absorbance at 790 and 710 nm. The absorbance difference was converted into Ca\(^{2+}\) using a standard curve (linear from 0–70 µM total Ca\(^{2+}\)).

**Ca\(^{2+}\)-stimulated ATPase activity.** Ca\(^{2+}\)-stimulated ATPase activity was determined using the enzyme-linked assay described by Luckin et al. (29). SR vesicles (30 µg) were placed in 1.5 ml of buffer (37°C) containing 100 mM KCl, 25 mM HEPES, 10 mM MgCl\(_2\), 1 mM NaCl, 0.2% NaN\(_3\), 5 µM lactate dehydrogenase (LDH), 7.5 U/ml PK, 2 µM CaCl\(_2\), 0.6 mM β-nicotinamide adenine dinucleotide, reduced form (NADH), 3 mM PEP, and 2 µM ionophore A-23187 (calcimycin) (pH 7.0). Basal, or Mg\(^{2+}\)-stimulated, activity was recorded for 3 min after the addition of 1 mM ATP. Total activity was recorded for 3 min after 2 µM CaCl\(_2\) was added. Ca\(^{2+}\)-stimulated ATPase activity was determined by subtracting basal from total activity. A diode array spectrophotometer (Hewlett Packard 8453) was used to follow absorbance at 340 nm. The rate of absorbance change was converted into rate of NADH utilization, using the extinction coefficient of NADH.

**SERCA, CK, and PK.** Content of the SERCA, CK, and PK were determined by using bovine serum albumin standards loaded onto SDS-PAGE with the purified SR and stained with Coomassie brilliant blue R-250. This technique was verified by using two other methods of calculating SERCA content. In the first, SERCA was purified from the sample preparation by using a technique modified from Coll and Murphy (8) and was used as a standard. In the second, SDS-PAGE and quantitative immunoblotting as described by Kandarian et al. (19) and Wu and Lytton (44) were used. Here, the SERCA standards were calibrated by determining the optical density (OD) of the SERCA band as a proportion of the OD of the entire lane. This proportion was then used to calculate the amount of SERCA loaded in each lane. A standard curve was then analyzed on the same SDS-PAGE and immunoblots as the samples. For the immunoblotting, a mini-gel system and an antibody for the SERCA 1a isofrom (IIH11, Affinity BioReagents) were used. All of these techniques resulted in identical estimations of SERCA content in the SR vesicles, and there was a linear relationship between the amount of protein added (5–20 µg) and the OD of each band either on the SDS-PAGE or the immunoblot (r\(^2\) = 0.99). Furthermore, we purified SR vesicles using the same methods as Kandarian et al. (19) to confirm our method of estimating SERCA yield. We found SERCA yields to be nearly identical to those of Kandarian et al. (19) (138.4 ± 13.4 µg/mg SR and ~145 µg/mg SR, respectively). The content of glycogen phosphorylase was determined by their OD on SDS-PAGE relative to the OD of a protein standard and presented as arbitrary units (AU). Glycogen phosphorylase content was determined semiquantitatively because it was not used to normalize SR Ca\(^{2+}\) uptake rates.

**SR-bound FITC.** FITC was used to determine E2/E1 equilibrium of SERCA. FITC maximum fluorescence emission was determined by using a method modified from Lalonde et al. (25). Because FITC is light sensitive, all procedures involved in FITC preparation and labeling (including all steps after the addition of FITC) were done in the dark. Briefly, SR vesicles (50 µg) and 350 µl of wash buffer were added to centrifugal filter units (30,000 normal molecular weight limit; Millipore) that had been prewashed once with the wash buffer. The wash buffer contained 0.1% NaN\(_3\), 5 mM HEPES, 0.1% Triton X-100, 0.1 mM AEBSF, 80 nM aprotinin, 2.2 nM leupeptin, 4 nM bestatin, 1.5 nM pepstatin A, and 1.4 nM E-64 (pH 7.0). The sample was incubated at room temperature, with shaking, for 10 min and then centrifuged for 20 min at 25°C (5,000 g). The flowthrough was discarded and 350 µl of labeling buffer containing 0.1% NaN\(_3\), 5 mM HEPES, 0.1% Triton X-100, 0.1 mM AEBSF, 80 nM aprotinin, 2.2 nM leupeptin, 4 nM bestatin, 1.5 nM pepstatin A, 1.4 nM E-64, and 2.5 µM FITC were added. The sample in the labeling buffer was incubated for 20 min, with shaking, at room temperature and then centrifuged for 20 min at 25°C (5,000 g). The flowthrough, as well as
the microcentrifuge tube, was discarded, and 350 μl of wash buffer were added to the filter unit and placed into a new microcentrifuge tube to wash away unbound FITC from the sample. The sample was gently vortexed and then centrifuged for 20 min at 25°C (5,000 g). The washing of unbound FITC step was repeated two additional times. The emission intensity of the sample was measured at 520 nm with an excitation wavelength of 490 nm. The emission intensity of the sample was quantified using FITC standards.

Statistics. The effects of no-EDTA and α-amylase on SERCA function was determined via two-way analyses of variance adjusted for repeated measures made on contralateral muscles taken from the same animal. Significance was set at $P < 0.05$.

RESULTS

Glycogen extraction from SR. The two methods of glycogen extraction used in the present investigation resulted in significant decreases in measured SR glycogen content. SR glycogen contents were 6.1 and 44.8% of controls in AM and EF groups, respectively ($P \leq 0.05$). EF SR glycogen content was found to be higher compared with AM ($P \leq 0.05$) (Fig. 1).

Glycogen phosphorylase content. As expected, SR glycogen extraction decreased glycogen phosphorylase associated with the SR. Amylase treatment did cause a significant decrease in SR glycogen phosphorylase content. SR glycogen phosphorylase content was reduced 12.2% of control in AM ($P \leq 0.05$). However, in the EF group, SR glycogen phosphorylase content was not reduced despite a significant reduction in SR glycogen concentration (Table 1). Representative lanes from SDS-PAGE for each treatment highlight the discrepancy in SR glycogen phosphorylase content (Fig. 2).

Purified SR protein profile. Another interesting consequence of glycogen extraction, especially in the AM group, was the change in the SR protein profile. Increases in the content of certain proteins associated with SR Ca$^{2+}$ uptake accompanied the loss of glycogen phosphorylase. These increases were also observed in the EF group in which glycogen phosphorylase was not found to be different. SERCA content was increased by 19.4% in AM SR ($P \leq 0.05$) (Fig. 2). Also, CK content was increased by 41.7 and 28.4%, and PK content was increased by 96.1 and 17.9% in AM and EF groups, respectively ($P \leq 0.05$) (Table 1).

Exogenous ATP supported SERCA function. Ca$^{2+}$-stimulated ATPase activity was increased in both AM and EF groups compared with control (mean control SR Ca$^{2+}$-stimulated ATPase activity was $2.41 \pm 0.23$ μmol ATP·mg SR protein$^{-1}$·min$^{-1}$) ($P \leq 0.05$). However, because SERCA content was increased in the AM group by 19.4%, Ca$^{2+}$-stimulated ATPase activities were normalized to SERCA content. After

Table 1. Protein contents for AM and EF glycogen-extracted groups and their respective controls

<table>
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<tr>
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<th>C-AM</th>
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<th>C-EF</th>
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<td>18.77±0.45*</td>
<td>8.08±0.43</td>
<td>9.51±0.29*</td>
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Protein contents were measured for amylase-treated (AM) and EDTA-free (EF) groups and their controls (C-AM and C-EF). GP, glycogen phosphorylase content expressed as micrograms of SERCA per milligrams of SR protein. CK, creatine kinase content expressed as micrograms of CK per milligrams of SR protein. PK, pyruvate kinase content expressed as micrograms of PK per milligrams of SR protein. Data are presented as the group means ± SE. *Significantly different from control ($n=6$).

Fig. 1. Sarcolemmal reticulum (SR) glycogen concentrations for both glycogen-extracted conditions (AM and EF) and their respective controls (C-AM and C-EF). Data are expressed in micrograms of glycogen per milligram of SR protein (error bars are SE). *Significantly different from control; #significantly different from the AM group ($n = 6$).

Fig. 2. Representative lanes from SDS-PAGE for each treatment. AM, amylase-treated sample; EF, sample homogenized and stored in an EDTA-free buffer; C, control group collected from the contralateral leg of the glycogen-extracted group; BSA STDS, bovine serum albumin standards; MW, molecular weight standards. A: bands containing sarco(endo)plasmic reticulum calcium ATPase (SERCA) and glycogen phosphorylase (GP) are indicated with arrows. B: bands containing pyruvate kinase (PK) and creatine kinase (CK) are indicated with arrows.
Ca\textsuperscript{2+}-stimulated ATPase activities were normalized to SERCA content, both the AM and EF groups remained elevated compared with C-AM and C-EF, respectively (P < 0.05) (Fig. 3).

**Exogenous ATP supported Ca\textsuperscript{2+} uptake.** Peak ATP-supported SR Ca\textsuperscript{2+} uptake rates were increased by 25.2% in the AM group compared with control (mean control SR Ca\textsuperscript{2+} uptake rate was 1.02 ± 0.05 nmol Ca\textsuperscript{2+}·mg SR protein\textsuperscript{−1}·min\textsuperscript{−1}) (P < 0.05). Peak ATP-supported SR Ca\textsuperscript{2+} uptake rates were not significantly different in the EF group compared with control. Because SERCA content was found to be higher in the AM group compared with control, peak ATP-supported SR Ca\textsuperscript{2+} uptake rates were normalized to SERCA content. Once normalized to SERCA content, there were no differences in the AM and EF groups compared with the C-AM and C-EF groups, respectively (Fig. 4).

**CK- and PK-supported Ca\textsuperscript{2+} uptake.** CK-supported SR Ca\textsuperscript{2+} uptake rates were increased under glycogen-extracted conditions. Peak CK-supported SR Ca\textsuperscript{2+} uptake rates were 23.6 and 18.9% higher in the AM and EF groups compared with C-AM and C-EF groups, respectively (mean control CK-supported SR Ca\textsuperscript{2+} uptake rate was 0.196 ± 0.004 nmol Ca\textsuperscript{2+}·mg SR protein\textsuperscript{−1}·min\textsuperscript{−1}) (P < 0.05) (Fig. 5). Similarly, PK-supported SR Ca\textsuperscript{2+} uptake rates were also increased under glycogen-extracted conditions. Peak PK-supported Ca\textsuperscript{2+} uptake rates were 17.9 and 17.5% higher in the AM and EF groups compared with C-AM and C-EF groups, respectively (mean control PK-supported SR Ca\textsuperscript{2+} uptake rate was 0.167 ± 0.004 nmol Ca\textsuperscript{2+}·mg SR protein\textsuperscript{−1}·min\textsuperscript{−1}) (P < 0.05) (Fig. 6). However, as in ATP-supported SR Ca\textsuperscript{2+} uptake, CK-supported and PK-supported uptake rates were normalized to SERCA. After normalizing to SERCA, only the EF group (14.5 and 13.3% for CK-supported and PK-supported SR Ca\textsuperscript{2+} uptake rates, respectively) remained elevated compared with C-EF (P < 0.05) (Figs. 5 and 6).

As stated earlier, both CK and PK contents were increased under glycogen-extracted conditions (Table 1). Therefore, SR Ca\textsuperscript{2+} uptake rates (normalized to SERCA) supported by endogenous CK and PK were again normalized to CK and PK.

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**Fig. 3.** Amylase-treated (AM) and EDTA-free (EF) glycogen-extracted SR Ca\textsuperscript{2+}-stimulated ATPase activities expressed as a percentage of control. Data were normalized both to SR protein and SERCA content (error bars are SE). *Significantly different from control (n = 6).

**Fig. 4.** AM and EF glycogen-extracted SR ATP-supported Ca\textsuperscript{2+} uptake rates expressed as a percentage of control. Data were normalized both to SR protein and SERCA content (error bars are SE). *Significantly different from control (n = 6).

**Fig. 5.** AM and EF glycogen-extracted SR CK-supported Ca\textsuperscript{2+} uptake expressed as a percentage of control. Data were normalized to SR protein, SERCA content, and SERCA and CK content (error bars are SE). *Significantly different from control (n = 6).
contents, respectively. Ca\textsuperscript{2+} uptake rates supported by endogenous CK and PK were 26.3 and 50.2% lower in the AM group compared with C-AM, respectively (P < 0.05). Although not significant, Ca\textsuperscript{2+} uptake rates supported by endogenous CK and PK were 9.3 and 6.1% lower in the EF group compared with C-EF, respectively (Figs. 5 and 6).

**FITC fluorescence emission.** Maximum FITC fluorescence emission was not different between glycogen-extracted groups and control groups. Even though decreases in fluorescence were not significant, FITC fluorescence emission was reduced by 26.8 and 9.7% in the AM and EF groups compared with C-AM and C-EF when data were normalized to SERCA, respectively.

**DISCUSSION**

In this investigation, purified SR was subjected to two different biochemical glycogen-extraction protocols. The results indicate that both α-amylase treatment and removal of EDTA from the homogenization and storage buffers lowered the amount of glycogen associated with the SR. When the data were normalized to SR protein content, neither of these treatments impaired Ca\textsuperscript{2+}-stimulated ATPase activity or SR Ca\textsuperscript{2+} uptake. In fact, these treatments caused apparent increases in Ca\textsuperscript{2+}-stimulated ATPase activity and SR Ca\textsuperscript{2+} uptake where either exogenous ATP was added or endogenous ATP was synthesized and utilized for SR Ca\textsuperscript{2+} transport. As expected, glycogen phosphorylase content was decreased as a result of AM glycogen extraction; however, a similar loss did not occur with the EF group. Interestingly, the content of many other proteins differed due to glycogen extraction as well. The AM group had a greater recovery of SERCA and a substantial loss of glycogen phosphorylase. CK and PK contents were increased as a result of both glycogen-extraction conditions. It was imperative to consider these altered protein contents while analyzing the data and assessing the effects of glycogen extraction on SR Ca\textsuperscript{2+} uptake. After being normalized to SERCA and either PK or CK content, AM glycogen extraction caused a lower SR Ca\textsuperscript{2+} uptake rate under endogenously synthesized ATP conditions.

**SR glycogen and glycogen phosphorylase.** We previously found that fatiguing muscular activity results in a large decrease in both glycogen and glycogen phosphorylase content associated with the SR (26). The glycogen concentrations of SR vesicles prepared from stimulated muscle were found to be only 5% of control. Both AM and EF treatments in the present investigation reduced SR glycogen concentration to 6.1 and 44.8% of control, respectively. Cuenda et al. (12) compared glycogen content of SR membranes purified from animals that were fasted for 48 h to those that were fed. Although they reported an approximate twofold decrease in SR glycogen content (data were not provided), their control values were dramatically lower than those reported by other investigators (14, 26, 27). Cuenda et al. (12) reported control SR glycogen content to be 32 μg of glycogen per milligram of SR membrane protein, whereas Entman et al. (14) reported a range of 300–700 μg of glycogen per milligram of SR membrane protein isolated from dog cardiac muscle. However, Cuenda et al. (12) purified SR from rabbit skeletal muscle and Entman et al. (14) purified SR from dog cardiac muscle. The glycogen concentration of the C-AM and C-EF groups in the present investigation were 455 and 367 μg of glycogen per milligram of SR protein, respectively. Differences between those reported by Cuenda et al. (12), those reported by Lees et al. (26, 27), and the present investigation may be explained in part by a more sensitive assay (27) and the presence of sucrose in the SR preparation. The SR preparation used in Cuenda et al. (12) had a large amount of sucrose in the storage buffer, which must be accounted for during the assay because it adds to the signal (27).

It is important to note that the magnitude of glycogen extraction was not directly proportional to the reduction of glycogen phosphorylase content. Glycogen phosphorylase was significantly reduced in the AM group to a level consistent with fatiguing stimulation (26). However, glycogen phosphorylase content was unchanged in the EF group compared with C-EF, even though SR glycogen content was reduced by more than 50%. These results are supported by other data collected in our lab using epinephrine and diet and exercise models of glycogen depletion. In these experiments, only extreme levels of glycogen depletion were associated with reduced SR glycogen phosphorylase content (unpublished data). Therefore, only large decreases in SR glycogen content result in dissociation of glycogen phosphorylase.

**SR SERCA, CK, and PK content.** An interesting outcome of glycogen extraction was an increase in SERCA, CK, and PK contents in the AM group, compared with C-AM. Also, CK and PK contents were higher in the EF group compared with C-EF. These changes were not anticipated, and no physiological mechanisms are proposed. However, because glycogen phosphorylase makes up a large proportion of the total SR protein in control samples, all remaining proteins must therefore make up a larger proportion of the total after glycogen phosphorylase is lost due to glycogen extraction (Fig. 2).
Certainly, it was important to account for these differences when analyzing SR Ca$^{2+}$ handling data. Normalizing data to protein content eliminated the artifact that glycogen extraction elevated SR Ca$^{2+}$ uptake.

**Glycogen extraction and SR function.** There are data suggesting that glycogen status may serve as a signal for SR Ca$^{2+}$ handling. Both Chin and Allen (10) and Stephenson et al. (38) reported a decline in SR Ca$^{2+}$ release as a result of glycogen extraction. Chin and Allen (10) used a muscle stimulation protocol to reduce cellular glycogen and a 60-min recovery, without glucose, to maintain glycogen depletion. Those investigators reported a sustained depression in measured [Ca$^{2+}$], during muscle contraction, compared with fibers that were allowed to recover in the presence of glucose. These data indicate that cellular glycogen may be important for optimal SR Ca$^{2+}$ release. In a similar study, Stephenson et al. (38) reduced glycogen stores from single-skinned muscle fibers (using a peeled sarcolemma preparation that leaves the T-tubular system intact). These investigators reported a decline in T-system activation-induced SR Ca$^{2+}$ release (a measure of excitation-contraction coupling).

**SR Ca$^{2+}$ uptake.** The most interesting finding in the present investigation is the decreased SR Ca$^{2+}$ uptake supported by CK and PK (normalized to protein content) as a result of glycogen extraction. There is evidence that the phosphocreatine circuit in skeletal muscle is subject to regulatory modulation in response to energy demand. Zoll et al. (47) investigated the effects of running training in rats on skeletal muscle mitochondrial function. These investigators reported dramatic increases in CK efficacy (131% increase in superficial plantaris fibers and 75% increase in deep plantaris fibers). CK efficacy was defined as the ratio between the apparent $K_m$ for ADP in the absence of creatine and in the presence of creatine. They concluded that this increase in CK efficacy allowed the trained animals to have better control of mitochondrial respiration by creatine, suggesting an improved linkage between energy utilization and production. Interestingly, skeletal muscle has a higher glycogen storage capacity as a result of training (see Ref. 37 for review). On the basis of these data, one might speculate that the phosphocreatine circuit might be sensitive to energy supply. Also, Korge and Campbell (23) reported a greater reduction in CK-supported SR Ca$^{2+}$ uptake rates measured in SR purified from fatigued muscles compared with those supported by ATP. Their data suggest that impaired SR Ca$^{2+}$ uptake is not solely explained by depressed SERCA function. Therefore, CK function may be under regulatory control and dictating SR Ca$^{2+}$ uptake in the intact cell. Although there are no data to indicate any mechanism(s) of regulation, it is interesting to speculate that energy supply, even glycogen status, may be involved. It is important to note that Korge and Campbell (23) did not report either CK or SERCA content.

Batts (3) used a glycogen-depletion model to assess the effects of reduced muscle glycogen on SR Ca$^{2+}$ handling and skeletal muscle function. Rats were fasted for 24 h, run for 90 min on a treadmill, and then separated into treatment groups. High-glycogen group rats were allowed standard rodent chow and a 5% sucrose solution (ad libitum), and low-glycogen group rats were only allowed access to water. This model reduced whole muscle glycogen by 42% and glycogen associated with the SR by about 90% in the low-glycogen group compared with the high-glycogen group. The reduction in SR glycogen was also associated with an increase of SERCA content as a proportion of total SR protein. Interestingly, this investigator reported decreased relaxation rates, which is consistent with decreased SR Ca$^{2+}$ uptake rates in the intact cell. Furthermore, these changes in muscle function were not accompanied by a decrease in the SR Ca$^{2+}$ uptake rates or Ca$^{2+}$-stimulated ATPase activities measured in purified SR. It is important to note that these purified SR measurements were made using exogenous ATP in the assays, whereas the in situ muscle function measurements are dependent on endogenous ATP synthesis. Thus exercise-induced SR dysfunction may be a result glycogen loss within the cell.

How does glycogen status affect endogenously supported SR Ca$^{2+}$ uptake? It is possible that the enzymes are directly affected by the conditions of their microenvironment. Molecular crowding is a term that describes the effect of high solute concentrations on chemical reactions by destabilizing either the reactants or products of a reaction, subsequently altering the equilibrium constants (for review see Ref. 30). Not only has PK been shown to be positively influenced by molecular crowding, but sucrose and polysaccharides are effective molecular crowding agents (4, 18, 28, 31, 32). Although the amount of solute (~100 mM) used in the study of molecular crowding is much higher than the endogenous glycogen (~1.5 mM in the assay medium) present with control SR samples in the Ca$^{2+}$ uptake assay system, it seems plausible to consider this concept. For instance, the glycogen associated with the SR may influence molecular crowding because it is localized at the enzyme system and not freely diffused in the assay medium. The volume of purified SR added to the assay medium is about 1% of the total volume. Therefore, the presence of ~1.5 mM glycogen localized at the SR may have a similar influence as ~100 mM freely diffusing macromolecular cosolute. This effect may be particularly important for the metabolic channeling involved in CK- and PK-supported Ca$^{2+}$ uptake.

**SR-bound FITC.** FITC exhibits increased fluorescence intensity when bound to the ATP-binding pocket of SERCA in the E2 conformation (33, 34). There is evidence that glycogen phosphorylase has an effect on SERCA conformation. Cuenda et al. (11) found the addition of exogenous glycogen phosphorylase (100–150 μg/ml or ~1–1.5 μM) to purified SR (devoid of endogenous glycogen phosphorylase) resulted in an increase in FITC florescence emission. These data suggest that glycogen phosphorylase causes a shift in the E2/E1 equilibrium to the E2 conformation of SERCA. Also, Luckin et al. (29) reported a 40% reduction in maximum FITC fluorescence emission along with a 40% decrease in Ca$^{2+}$-activated ATPase activity in SR samples purified from fatigued muscle, compared with control. These data indicate that lowered SR Ca$^{2+}$ uptake rates normally found in fatigue may be related to a shift in the E2/E1 equilibrium to the E2 conformation of SERCA. In the present investigation, only the AM group displayed decreased glycogen phosphorylase content. Although not significant, FITC fluorescence emission normalized to SERCA content was reduced by 26.8% in the AM group compared with C-AM. Fittingly, FITC fluorescence emission normalized to SERCA content was only reduced by 9.7% in the EF group, compared with C-EF (also not significant). If, in fact, reduced FITC fluorescence emission in the AM group was physiologically significant, even though not statistically significant, then
why didn’t ATP-supported measures of SERCA function reflect this? One possible explanation involves the physiological relevance of exogenously added ATP used to measure these processes. When excess amounts of energy substrate are added to measure peak enzyme activity and Ca$^{2+}$ transport, changes in normal cellular mechanisms involved in Ca$^{2+}$ transport (e.g., metabolic channeling and compartmentation) are not accounted for. For instance, processes involved in localized ATP synthesis in cellular compartments, as well as the channeling characteristics between enzymes, are ignored when excess exogenous ATP is added to an assay.

The results from the present investigation support the hypothesis that glycogen extraction has a detrimental effect on SR Ca$^{2+}$ uptake when supported by endogenously synthesized ATP and normalized to SERCA and to either PK or CK content. It was vital that these data be normalized to protein content because altered protein yields were a consequence of glycogen extraction. AM and EF groups displayed two distinct degrees of glycogen extraction. The magnitude of glycogen extraction seemed to be reflected in the changes in CK- and PK-supported SR Ca$^{2+}$ uptake and in the loss of glycogen phosphorylase. In these measurements, EF glycogen extraction seemed to be reaccounted for. For instance, processes involved in localized ATP synthesis in cellular compartments, as well as the channeling characteristics between enzymes, are ignored when excess exogenous ATP is added to an assay.

It is entirely possible that the SR relies heavily on glycolysis-supported ATP synthesis and the phosphorelay circuit in vivo. Therefore, metabolic channeling experiments, like CK- and PK-supported SR Ca$^{2+}$ uptake, may be more physiologically important than the measurement of Ca$^{2+}$ transport supported by exogenous ATP. Altered Ca$^{2+}$ transport properties of the SR were only detected in the metabolic channeling systems in which the SERCA utilizes endogenous ATP. Changes in endogenously supported SR Ca$^{2+}$ uptake due to glycogen extraction potentially affected the source of ATP synthesis (either PK or CK) and the effectiveness of energy utilization for Ca$^{2+}$-transport (SERCA) or altered the metabolic channeling properties. This experiment highlights the importance of normalizing measurements to the enzymes of interest instead of total protein. Moreover, the measurement of physiologically important processes is vital to the understanding of altered physiological function found in fatigu.

GRANTS

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