CHAPTER 3: Experimental Design and Methods

Specific Aims

The specific aims of the present investigation were the following:

1. To determine if glycogen associated with the SR is decreased following fatiguing stimulation.
2. To determine if glycogen phosphorylase content of the SR is decreased following fatiguing stimulation. The loss of glycogen phosphorylase was measured by three methods: the amount of PLP associated with the SR, optical density of the glycogen phosphorylase band on SDS-PAGE, and glycogen phosphorylase activity assay.
3. To determine if decreased SR glycogen concentration and glycogen phosphorylase content and activity are accompanied by decreased maximum SR Ca^{2+} uptake rate.

Experimental Design

In this experiment, three separate groups of rats had to be used. One group was used for whole muscle glycogen concentration (gastrocnemius and plantaris complex), and two other groups were used for measurement of variables associated with the SR vesicles (made from the gastrocnemius and plantaris complex). All of the measurements, except whole muscle glycogen, were made on

the SR isolated from these two groups of animals. The only differences between these two groups were that SR Ca^{2+} uptake measurements had to be made using SR vesicles stored in 300 mM sucrose (sucrose serves as a cryo-protectant for maintenance of SR membrane integrity) and SR glycogen concentration had to be measured using SR vesicles stored without sucrose (sucrose interferes with the measurement of glycogen). All remaining measurements made using the SR vesicles could be made using either of these two groups.

One leg of each animal was subjected to the treatment and the contralateral leg served as the control. This allowed for the paired statistical analyses. The treatment consisted of repetitive electrical stimulation of the sciatic nerve. Stimulation of the sciatic nerve results in contraction of all the muscles innervated by that nerve, including the gastrocnemius and plantaris muscles. The stimulation protocol entailed contractions elicited once per second for 15 minutes. The experimental groups and measurements are shown in the Table 1.

	Group 1	
	Muscles homogenized in perchloric acid	
	Control Muscle	Fatigued Muscle
Measurements	Whole muscle glycogen concentration	

	Group 2	
	SR isolated and stored in 300 mM sucrose	
	Control Muscle	Fatigued Muscle
Measurements	SR calcium uptake SR PLP concentration SR phosphorylase content (SDS-PAGE) SR phosphorylase activity SR calcium ATPase content (SDS-PAGE)	

	Group 3	
	SR isolated and stored without sucrose	
	Control Muscle	Fatigued Muscle
Measurements	SR glycogen concentration SR PLP concentration SR phosphorylase content (SDS-PAGE) SR phosphorylase activity SR calcium ATPase content (SDS-PAGE)	

Table 1. The experimental groups and measurements made based on the method of tissue collection and storage.

Animals

All procedures were approved by the Animal Use and Care Committee of Virginia Tech. Female Sprague-Dawley rats (200-225g) were housed at the Virginia Tech Laboratory Animal Resource Facility. Animals were fed *ad libitum* (Purina Rodent Laboratory Chow), allowed free access to water, and exposed to a 12 hour light:dark cycle.

Stimulation Protocol

The gastrocnemius and plantaris muscles from female Sprague-Dawley rats were used for all the experiments. One leg was stimulated (fatigue) and the contralateral leg served as the control (rested). Animals were anesthetized with sodium pentabarbitol (90 mg/kg body weight). The sciatic nerve was surgically exposed so that the stimulation electrode could be attached, without damaging the nerve. To expose the sciatic nerve, first the skin of the hind leg was completely removed. Second, the biceps femoris muscle was landmarked on the lateral aspect of the upper hind leg. Third, while lifting the muscle, an incision was made (about one third of the length of the muscle from the origin) perpendicular to the length of the biceps femoris muscle. Once the outer musculature was cut away, the sciatic nerve could be visualized. The

surrounding connective tissue was cut so that forceps could be passed under the nerve. This was done so that the stimulation electrode could be easily attached.

The next phase of the surgery served to secure the distal portion of the muscles. Surgical suture was tied and glued at the insertion of the tendo calcaneus before the distal portion of the calcaneus was cut with bone cutters. The soleus muscle was cut at its insertion as to prevent it from contributing to force production.

Finally, the whole animal was placed on a custom built apparatus, so that its knee could be clamped to prevent movement, while the stimulation protocol was being performed. The free end of the surgical suture was attached to an isometric transducer. Ringer solution filled the chamber of the apparatus, covering the exposed portion of the leg, then a few drops of mineral oil were placed around the exposed sciatic nerve. The ringer solution was warmed to about 37˚ C prior to the surgery and the temperature was maintained using a heat lamp. The ringer solution (pH 7) contained the following: 135 mM sodium chloride (NaCl), 5 mM potassium chloride (KCl), 2.5 mM calcium chloride ($CaCl_2$), 1 mM magnesium sulfate ($MgSO_4$),

1 mM sodium phosphate (NaH_2PO_4), and 15 mM sodium bicarbonate ($NaHCO_3$). Tetanic contractions were elicited using a Grass S48 stimulator and force measurements were measured, displayed, and recorded using a Harvard apparatus isometric transducer, Tecktronix 2201 oscilloscope and Harvard chart recorder. The fatigue protocol entailed *in situ* stimulation for 15 minutes via the sciatic nerve. Contractions were elicited by 100 msec trains of pulses (75 Hz) delivered once per second.

Whole Muscle Glycogen

Control and fatigue gastrocnemius/plantaris muscles were rapidly removed and homogenized with three, 15 second bouts (VirTis VirTishear) in 5 ml of 0.6 M perchloric acid solution per gram of muscle wet weight then frozen and stored at –80˚C. Glycogen content was then determined by the method described by Keppler and Decker (1984). A small amount of sample (0.2 ml) was incubated in an acidic solution (174 mmol/l acetic acid containing glucoamylase 8.7 kU/l) for 2 hours at 30-50˚C (pH 4.8). After the incubation, 1.0 ml of perchloric acid solution (0.6 M) was added, mixed, and centrifuged for 10 minutes at 1600 x g. 50 µl of the resulting supernatant was removed and added to 1 ml of an enzymatic buffer containing the following: 0.3 M

triethanolamine (TEA), 4 mM magnesium sulfate ($MgSO_4$), 120 mM potassium hydroxide (KOH), 1 mM adenosine triphosphate (ATP), 0.9 mM beta-nicotinamide adenine dinucleotide phosphate (β-$NADP^+$), and 700 U/l glucose-6-phosphate dehydrogenase (G-6-P DH). The absorbance (A_1) was measured after 5 minutes at 365 nm to determine the amount of glucose-6-phosphate (G-6-P). 5 µl of hexokinase ($\geq$ 280 kU/l) suspension was then added to each cuvette and a second absorbance (A_2) was measured after 10 minutes. This absorbance determined the total amount of glucose and G-6-P. In order to determine total glucose content (both free tissue glucose and glucose derived from the digestion of glycogen), A_1 was subtracted from A_2. Another 0.2 ml of sample was used to determine free tissue glucose without glycogen digestion. This sample was centrifuged at high speed in a micro-centrifuge. 50 µl of the resulting supernatant was drawn off and added to 1 ml of the same enzymatic buffer. The first absorbance (A_1) was measured to determine the amount of G-6-P, 5 µl of hexokinase suspension was added, and then A_2 was measured after 10 minutes to determine the amount of glucose and G-6-P. A_1 was subtracted from A_2 to determine tissue glucose concentration. The tissue glucose concentration was subtracted from the total glucose concentration (including

that digested from glycogen) in order to determine muscle glycogen. Glycogen standards were prepared from purified rabbit liver.

SR Vesicle Preparation

SR vesicles were prepared by the method previously described by Williams et al. (1998). Control and fatigue gastrocnemius/plantaris muscles were homogenized in 5 volumes of buffer (5 ml per gram of muscle wet weight) (pH 6.8) containing 20 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethane-sulfonic acid] (HEPES), 0.2% sodium azide (NaN_3), and 0.2 mM phenylmethylsulfonylfluoride (PMSF). These samples were centrifuged at 8,000 x g for 15 minutes in an International Equipment Company B-22 centrifuge. The supernatant was then poured through 4 layers of gauze. 0.0447 g potassium chloride (KCl)/ml of supernatant was added to each sample for a final KCl concentration of 600 mM, and then centrifuged at 12,000 x g for 45 minutes. The supernatant was drawn off and centrifuged again at 49,000 x g for 60 minutes. The pellet from the last centrifugation, containing the isolated SR proteins and membranes, was resuspended, frozen at –80˚ C, and stored for later use in the a storage buffer (pH 6.8) containing the following: 20 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethane-sulfonic

acid] (HEPES), 0.2% NaN_3, 0.2 mM PMSF, and 150 mM KCl. It is important to note that SR vesicles used for SR Ca^{2+} uptake measurements were stored in a storage buffer containing the following: 20 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethane-sulfonic acid] (HEPES), 0.2% NaN_3, 0.2 mM PMSF, 150 mM KCl, and 300 mM sucrose.

Protein Concentration Assay

The protein concentration of the resuspended SR vesicles was determined using the Bradford dye-binding assay adapted by Bio-Rad using bovine serum albumin as a standard (Bradford, 1976). The binding of basic and aromatic amino acid residues to Coomassie® Brilliant Blue G-250 dye shifts the maximum absorbance from 465 nm to 595 nm (Sedmack and Grossberg, 1977; Reisner et al., 1975; and Fazakes de St. Groth et al., 1963).

SR Glycogen

Glycogen associated with the SR was measured by digesting the glycogen and measuring the glucose molecules enzymatically. Resuspended SR (a volume containing 50 μg of protein) was incubated in 0.5 ml of an acetic acid (174 mmol/l) solution containing glucoamylase (8.7 kU/l) for 2 hours at 30-50˚C (pH 4.8). 50 μl of each sample was then

added to 1 ml of a buffer (pH 7.5) containing the following: 0.3 M TEA (triethanolamine), 4 mM magnesium sulfate ($MgSO_4$), 120 mM potassium hydroxide (KOH), 1 mM adenosine triphosphate (ATP), 0.9 mM beta-nicotinamide adenosine dinucleotide phosphate (β-$NADP^+$), 700 U/l glucose-6-phosphate dehydrogenase (G-6-P DH), and hexokinase (1.3 kU/l). After 10 minutes of incubation at room temperature, fluorescent emission of the reduced form of beta-nicotinamide adenine dinucleotide phosphate (β-NADPH) was measured at 450 nm while the excitation wavelength was set at 365 nm. Glycogen standards were prepared using glycogen purified from rabbit liver.

Glycogen Phosphorylase Activity

Glycogen phosphorylase activity, measured in the direction of glycogen breakdown, was done using a method adapted from Chi et al. (1983). 500 ng of each SR sample was added to 100μl of reagent (pH 7.0) containing the following: 50 mM imidazole, 50 mM glycogen, 20 mM potassium phosphate (K_2HPO_4), 0.5 mM magnesium chloride ($MgCl_2$), 1 mM 5′-adenosine monophosphate (5′-AMP), 2 μM glucose-1,6-bisphosphate (G-1,6-biP), 0.5 mM dithiothreitol (DTT), 0.25% bovine serum albumin (BSA), and 0.4 U/ml phosphoglucomutase (PGM). Because of the known difference

between glycogen phosphorylase content, control samples were allowed to react for 15 minutes at room temperature, while fatigue samples were allowed to react for 1 hour at room temperature. 10 μl of 0.5 N HCl was added to each sample in order to stop the reaction. They were then allowed to sit for 10 minutes at room temperature. 1 ml of a second reagent was added to the product of the first reaction. The second reagent contained the following: 50 mM Tris-HCl, 100 μM beta-nicotinamide adenine dinucleotide phosphate (β-NADP), 1 mM ethylenedianminetetraaacetic acid (EDTA), 1 U/ml glucose-6-phosphate dehydrogenase (G-6-P DH), and 0.35 U/ml phosphoglucoisomerase. The samples were allowed to react in the second reagent for 20 minutes and then the fluorescent emission of the reduced form of beta-nicotinamide adenine dinucleotide phosphate (β-NADPH) was measured at 450 nm while the excitation wavelength was set at 365 nm. Standards used were 0, 5, 10, 25, and 35 nmoles/5μl of glucose-1-phosphate.

PLP Assay

Pryidoxal 5-phosphate (PLP) associated with the SR vesicles was measured using a method modified from Ubbink et al. (1985), Mahuren et al. (1990), Cordes et al. (1962),

and Gregory (1980). By removing and measuring PLP, the glycogen phosphorylase content can be determined since over 95% of PLP in skeletal muscle is bound to this enzyme. First, the sample had to be prepared before it was injected into the high performance liquid chromatography (HPLC) system. To prevent PLP degradation, samples were prepared under yellow light. 0.5 ml of 10% trichloroacetic acid (TCA) was added to 1 ml of sample (containing 200 μg of SR protein). The precipitated protein was then pelleted by centrifuging the samples at 1000 x g for 10 minutes. The supernatant was then removed and 50 μl of 0.5 M semicarbazide was added to each sample. The samples were then incubated for 15 minutes at 40˚ C. Diethyl ether (3 ml) was added to each sample, vortexed, and removed (after centrifugation at 1000 x g for 10 minutes). This last step was repeated. Dichloromethane (3 ml) was then added to each sample, vortexed and then the supernatant was removed and saved (after centrifugation at 1000 x g for 10 minutes). 100 μl of this supernatant was injected into the HPLC system (Waters™) and run through an ODS C18 4.6 mm x 25 cm Zorbax column from Mac-Mod Analytical with a mobile phase buffer. The mobile phase buffer was prepared in the following manner: 0.05 M potassium phosphate (KH_2PO_4) and 8% (volume/volume) acetonitrile were mixed in water, and pH

adjusted to 2.9 with o-phosphoric acid. The mobile phase buffer was then filtered and degassed. The mobile phase was run at a pressure no greater than 2200 pounds per square inch (PSI) at a rate of 1.1 ml/minute. Fluorescent emission of PLP was measured at 478 nm, while the excitation wavelength was 367 nm. The PLP peak on the fluorescent emission output was detected by its known retention time (~3.4 minutes) through this particular column using Waters Millennium 32 Chromatography Manager. Fluorescent emission was enhanced via a post-column bisulfite buffer containing 4% sodium hydroxide (NaOH) in HPLC water, which was filtered and degassed before use. Standards were made from a stock solution containing 10 mg of PLP in 1 l of HPLC water. Standards were diluted down to 0, 0.5, 1, 5, 10, and 20 ng/100 µl of PLP.

SDS-PAGE

Samples were prepared by first diluting (1 µg/µl SR protein) with water and then combining them with sample buffer 1:1 (volume:volume). 25 ml of sample buffer was prepared by mixing 0.25 ml beta-mercaptoethanol (β-MEOH), 1 g sodium dodecyl sulfate (SDS), 4 ml of 1 M Tris (ph 6.8), 5 ml glycerol, 0.5 g bromophenol blue, and bring to 25 ml with d H_2O. The samples in the sample buffer were placed in

glass test tubes, boiled for 2 minutes, tops were wrapped with parafilm, and then frozen at –80˚ C until they were needed. The final concentration of the samples in the sample buffer was 0.5 µg/µl.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970). Gels were run on a mini-PROTEAN® II cell from Bio-Rad. The 5% acrylamide separating gel was prepared by mixing 12.3 ml H_2O, 5 ml 1.5 M Tris (pH 8.8), 0.2 ml of 10% SDS, and 2.5 ml of 40% acrylamide. The separating gel was then placed in a vacuum for 20 minutes. 20 µl of 97% (electrophoresis grade) N,N,N’,N’-tetramethylethylenediamine (TEMED) and 100 µl of 10% ammonium persulfate were added to the separating gel, gently mixed, poured into the mini-PROTEAN® II cell (1 cm below the comb), and 20% ethanol was overlayed. The separating gel was allowed to polymerize for 30 minutes. After polymerization, the ethanol was poured off, washed from between the glass plates with 0.1% SDS, and then filter paper was used to dry in between the glass plates. The 4% acrylamide stacking gel, prepared at the same time as the separating gel, contained 12.68 ml H_2O, 0.5 M Tris (pH 8.8), 0.02 ml of 10% SDS, and 2 ml of 40% acrylamide.

After mixing the components, the stacking gel was placed in a vacuum for 20 minutes. 20 µl of 97% (electrophoresis grade) TEMED and 100 µl of 10% ammonium persulfate were added to the stacking gel, gently mixed, poured into the mini-PROTEAN® II cell, and lane combs were placed in between the two glass plates. The stacking gel was allowed to polymerize for 30 minutes. After polymerization, the combs were gently pulled out and electrode buffer (pH 8.3) containing 3 g/l Tris base, 24 g/l glycine, and 3 g/l SDS was poured into the upper chamber. The lanes were washed out with the electrode buffer and then loaded with 10 µg of SR protein in sample buffer. Electrode buffer was then poured into the lower chamber and the power supply was connected. The running conditions were set at 45 mA (constant), until the tracking dye ran off the gel. Gels were stained overnight in a solution containing the following: 0.1% Coomassie Blue R-250, 40% methanol, and 10% acetic acid. After staining, the gels were destained for about 1 hour in a solution containing: 50% H_2O, 40% methanol, and 10% acetic acid. The bands corresponding to the molecular weight of SR Ca^{2+}-ATPase and glycogen phosphorylase were scanned using MultiImage™ Light Cabinet from Alpha Innotech Corporation and analyzed using AlphaImager™ 2000 Documentation & Analysis System. From

these scanned images, optical density of these bands were determined.

SR Ca^{2+} Uptake

The ability of the SR vesicles to regulate Ca^{2+} concentration was measured using a Jasco CAF-110 intra-cellular ion analyzer with a 75 watt xenon high-pressure lamp. Extravesicular Ca^{2+} was measured fluorometrically using the Ca^{2+} indicator Fura-2 (excitation wavelengths 340 nm and 380 nm; emission wavelength 500 nm). Free Ca^{2+} concentration ($[Ca^{2+}]_{free}$) was calculated by measuring the ratio (R) of the emissions at 500 nm from both excitation wavelengths and using the following equation: $[Ca^{2+}]_{free} = K_d \times \beta \times (R - R_{min})/(R - R_{max})$, where the Fura-2 dissociation constant (K_d) was 70 nM, R_{min} and R_{max} are the R values measured in the Ca^{2+} uptake buffer in a Ca^{2+} free medium and a Ca^{2+} saturated medium, respectively, and β is the fluorescence ratio of EGTA- and Ca^{2+}-supplemented buffers at 340 nm (Grynkiewicz et al., 1985). 50 μg of SR protein was added to 1 ml of Ca^{2+} uptake buffer containing the following: 100 mM potassium chloride (KCl), 20 mM N-[2-hydroxyethyl] piperazine-N’-[2-ethane-sulfonic acid] (HEPES), 1 mM magnesium chloride ($MgCl_2$), 5 mM potassium oxalate, and 1 mM adenosine triphosphate (ATP). Then, 10 μl

of FURA-2 (for a final Fura-2 concentration of 2 μM) was added to the uptake buffer with the SR protein and allowed to equilibrate at 37˚ C until the fluorescence ratio remained constant. After initiating the data collection program, 1 μl of a 40 mM $CaCl_2$ stock solution was added to the uptake buffer. Fluorescence ratio values were sampled at 2 Hz using Labtech Notebook pro and saved to disk for later analysis. Ca^{2+} uptake rates were computed from the steepest negative slopes after the addition of $CaCl_2$.

Statistics

One tailed paired t-tests were used for comparisons between control and fatigue conditions of all variables except Ca^{2+}-ATPase optical density from the SDS-PAGE. A two tailed paired t-test was used for the optical density of Ca^{2+}-ATPase comparison because a direction for any potential difference was not apparent from the literature. Significance was set at the $P \leq 0.05$ level of confidence.

CHAPTER 4: Results

The effect of stimulation on muscle force production

Force production by the gastrocnemius and plantaris muscles was measured at the beginning (control) and end (fatigue) of the 15 minute stimulation protocol. Typically, force dropped off rapidly over the first minute of stimulation, followed by a slow and steady decline over the remaining time. At the end of the 15 minute protocol, fatigued muscle force production was reduced to 29.6% of control.

The effect of stimulation on whole muscle and SR glycogen

Glycogen concentrations for whole muscle and SR preparations are shown in Figure 3. Whole muscle glycogen concentration was significantly decreased to 22.9% of control from 7.03 ± 0.28 to 1.61 ± 0.20 µg/mg wet weight ($p \leq 0.05$). These data agree with Spriet et al. (1989). Because glycogen associated with the SR had not previously been measured in our laboratory, pilot data was collected using two different assays. The results from these data are outlined in appendix B. Glycogen associated with SR was significantly reduced to 5.1% of control from 415.39 ±

76.62 to 20.36 ± 2.07 μg/mg SR protein ($p \leq 0.05$). Total SR glycogen recovered from the isolation procedure was 0.235 ± 0.043 μg/mg wet wt. in control samples. It is important to note that even though total recovered SR glycogen only accounts for a small portion of the whole muscle glycogen, this is likely due in part to the fact that much of the SR is lost in the first and second pellets during the isolation.

The effect of stimulation on glycogen phosphorylase associated with the SR

Optical density of glycogen phosphorylase determined via SDS-PAGE stained with Coomassie blue is shown in Figure 4. Glycogen phosphorylase content was significantly reduced to 21.2% of control from 0.96 ± 0.062 to 0.204 ± 0.025 arbitrary units ($p \leq 0.05$) (Figure 5).

Glycogen phosphorylase activity of the SR for both control and fatigue are shown in Table 2. Activity of glycogen phosphorylase in the direction of glycogen breakdown was significantly reduced to 4.1% of control from 2.8 ± 0.16 to 0.115 ± 0.018 nmol G-1-P/minute per μg SR protein ($p \leq 0.05$). Decreased glycogen phosphorylase activity

is likely due to less glycogen phosphorylase present in the sample.

Pryidoxal 5-phosphate (PLP) concentrations of the SR for both control and fatigue are shown in Table 2. PLP concentration was significantly reduced to 3.3% of control from 641.27 ± 121.45 to 21.27 ± 5.88 ng/mg SR protein ($p \leq 0.05$).

The effect of stimulation on SR Ca^{2+} Uptake

Maximum Ca^{2+} uptake rates of SR were significantly reduced to 80.8% of control from 2.99 ± 0.21 to 2.42 ± 0.205 ug/mg/min ($p \leq 0.05$). Optical density of SR Ca^{2+}-ATPase determined by SDS-PAGE stained with Coomassie blue is shown in Figure 4. There was no significant difference in optical density of SR Ca^{2+}-ATPase between control (0.849 ± 0.057 arbitrary units) and fatigue (0.770 ± 0.06 arbitrary units) SR preparations ($p > 0.05$). Summary of the effect of prolonged muscle contraction on all the variables measured in the present investigation expressed as a percent of control is shown in Figure 6.

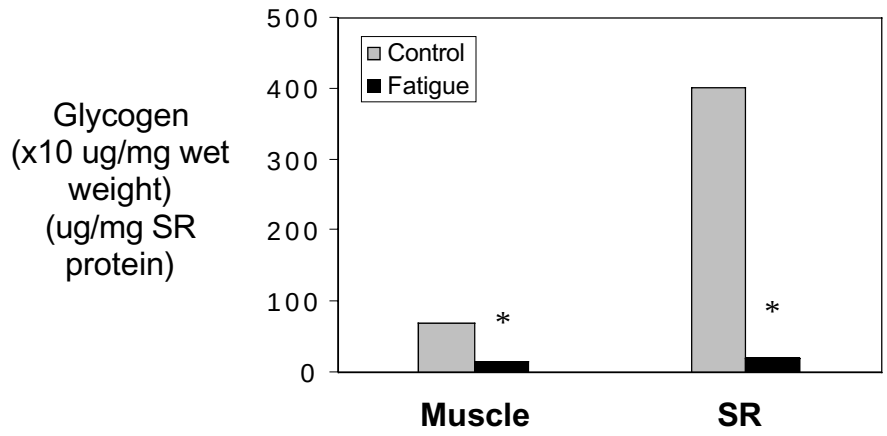

Figure 3. Whole muscle and SR glycogen concentration for control and fatigue conditions. Muscle: whole muscle glycogen concentration (x10 µg/mg muscle wet weight). SR: SR glycogen concentration (µg/mg SR protein). * Denotes significant differences compared to control ($p \leq 0.05$).

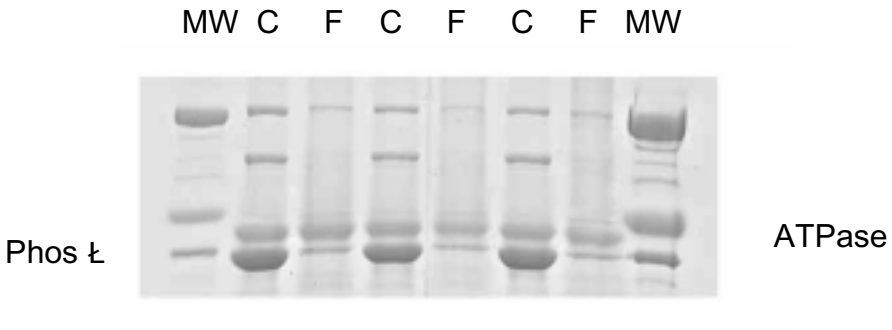

Figure 4. Representative SDS-PAGE with the bands containing Phos: glycogen phosphorylase; and ATPase: SR Ca^{2+} ATPase. The lanes are labeled MW: molecular weight marker; C: control; F: fatigue.

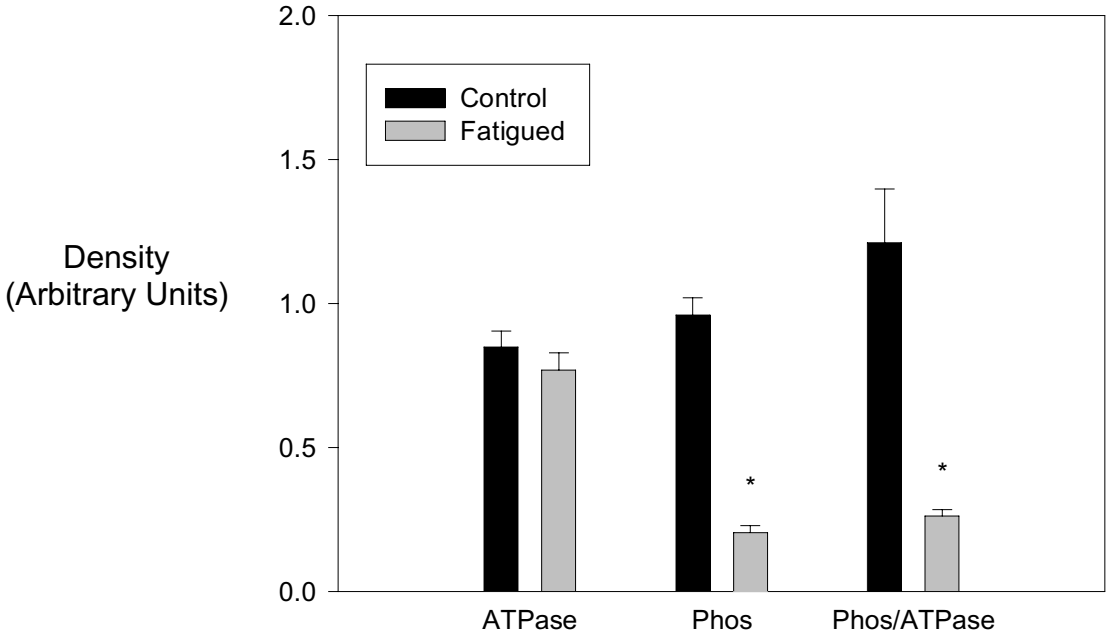

Figure 5. ATPase: SR Ca^{2+}-ATPase optical density with the bands labled on the representative scanned gel above. Phos: glycogen phosphoylase optical density with the 97 kD glycogen phosphorylase marker labled on the representative scanned gel above. Phos/ATPase: the ratio of glycogen phosphorylase and SR Ca^{2+}-ATPase for control and fatigue conditions. * denotes significant ($p \leq 0.05$) difference from control.

Table 2. Glycogen phosphorylase (Phos) activity and pryidoxal 5-phosphate (PLP) concentration associated with the SR. * denotes significant difference ($p \leq 0.05$) compared to control.

Condition	Phos Activity (nmol G-1-P/min. per μg SR protein)	PLP Concentration (ng/mg SR protein)
Control	2.80 ± 0.16	641.27 ± 121.45
Fatigue	0.115 ± 0.018 *	21.27 ± 5.88 *

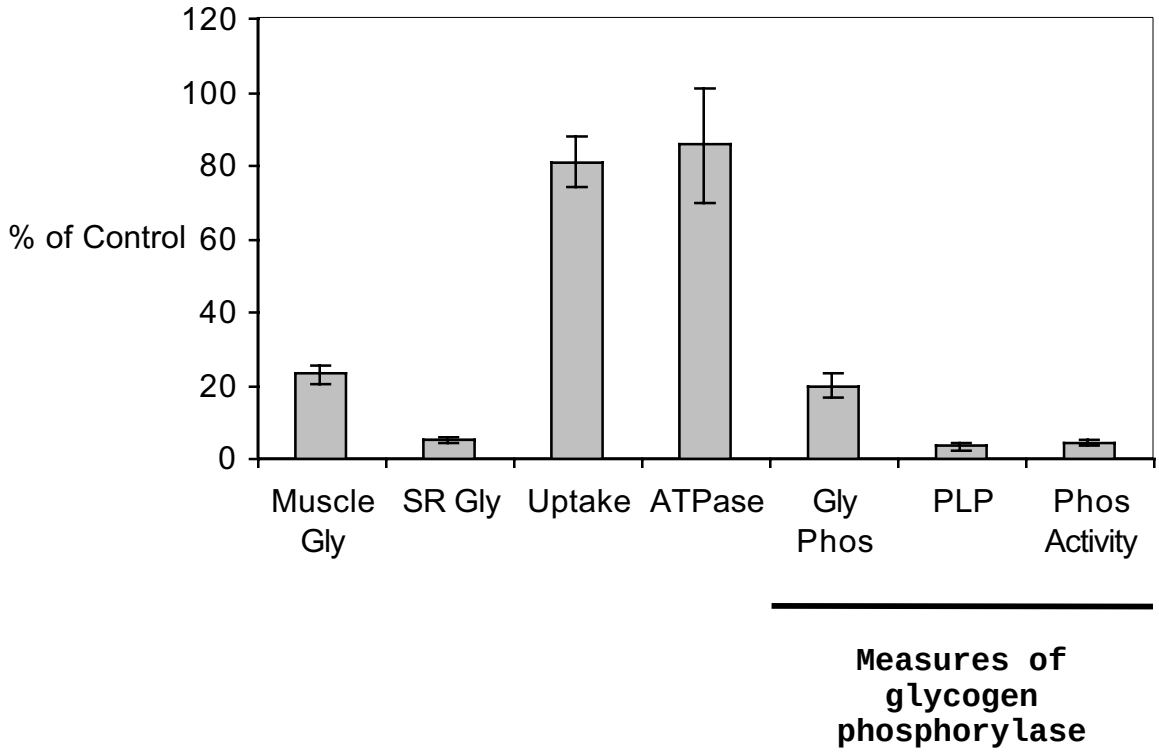

Figure 6. Summary of the changes due to fatigue relative to control values. Muscle Gly: whole muscle glycogen concentration; SR Gly: glycogen concentration associated with the sarcoplasmic reticulum; Uptake: maximum calcium uptake rate associated with the sarcoplasmic reticulum; ATPase: optical density of the band containing SR calcium ATPase on a SDS-PAGE; Gly Phos: optical density of the band containing glycogen phosphorylase on a SDS-PAGE; PLP: pryidoxyl 5-phosphate concentration associated with the sarcoplasmic reticulum; Phos Activity: glycogen phosphorylase activity associated with the sarcoplasmic reticulum. Error bars are expressed as ± SEM. * denotes significant difference compared to control ($p \leq 0.05$).