CHAPTER 5: Discussion

Discussion

In this study the effects of prolonged muscle contraction on glycogen, glycogen phosphorylase, and maximum Ca^{2+} uptake rates associated with the SR were investigated. The sciatic nerve from one leg of animals was repetitively stimulated in order to cause fatigue of the gastrocnemius/plantaris muscles. These contractions were elicited once per second for 15 minutes. This protocol reduced force to less than 30% of initial, therefore, these stimulated muscles were considered to be fatigued. The contralateral leg of each animal served as the control. Significant differences were found for glycogen, glycogen phosphorylase, and maximum Ca^{2+} uptake associated with the SR between control and fatigued muscles.

Whole muscle glycogen concentration was significantly decreased to 22.9% of control as a result of *in situ* stimulation (Figure 2). These data agree strongly with the results from other investigators (Stephenson et al., 1999; Chin et al., 1997; Kelso et al. 1987; Galbo et al., 1979; Ahlborg et al., 1967; and Bergström et al., 1967). Decreases in SR glycogen were accompanied by a reduction in maximum Ca^{2+} uptake rates of the SR to 80.8% of control ($p \leq 0.05$). Glycogen associated with light SR was significantly reduced to 5.1% of control ($p \leq 0.05$) (Figure 2). These results are not surprising because glycogen is an important substrate for the production of ATP, however, this has not previously been shown. In 1989, Fridén et al. used periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP) staining of ultra-thin muscle sections from human vastus lateralis. The staining revealed a compartmental distribution of glycogen within the muscle cell. After exercise (sixty, 8 second sprint cycling bouts), glycogen depletion was particularly noticeable at the N_2-line, or the lateral portion of the I-band. It is important to note that the SR has been shown to be present at the I-band (Sigel et al., 1969; and Pette 1975).

Glycogen associated with the SR in control samples was determined to be approximately 400 μg/mg SR protein in the present investigation. Previous investigations by Cuenda et al. (1994) and Entman et al. (1976) reported SR glycogen concentrations to be 32 and 300 to 700 μg/mg SR protein, respectively. The discrepancy between these two previous experiments can be explained by looking at the type of tissue collected. Cuenda et al. (1994) isolated SR from rat skeletal muscle, while Entman et al. (1976) performed their experiment on SR isolated from dog cardiac muscle. The discrepancy between the reported SR glycogen concentration in present investigation and that from Cuenda et al. (1994) is likely due to SR storage conditions and the SR glycogen assay. First, it appears that Cuenda et al. (1994) and Entman et al. (1976) both had sucrose present in their SR preparations. Because sucrose is measured as if it were glycogen in their assay, it must be subtracted from the measurement if it is present. Since the amount of glycogen associated with the SR is very small compared to the sucrose content, this can be problematic. It is unclear whether sucrose had to be subtracted from their analyses or if some other storage method was used for both of these investigations. Second, both Cuenda et al. (1994) and Entman et al. (1976) used an assay that utilized sulphuric acid to hydrolyze the glycogen and phenol for colorization, followed by measuring the absorbance change. The present investigation utilized an assay that used amyloglucosidase to hydrolyze the glycogen, and fluorometric measurement of β-NADPH in an enzymatic system. The latter assay has been shown to be a more sensitive method for the measurement of glycogen associated with the SR (Lees et al., 2000; for a brief summary, see appendix

B). There are two types of glycogen normally found in skeletal muscle: proglycogen and macroglycogen. Macroglycogen can be characterized by its acid solubility. Since there is a perchoric acid precipitaion of the protein in the phenol-sulfuric acid assay and glycogen is measured from the resulting pellet, it is possible that this assay does not measure any of the macroglycogen associated with the sample.

Glycogen phosphorylase has been shown to be associated with the SR (Entman et al., 1980; and Wanson et al., 1972). Specifically, glycogen phosphorylase associated with the SR is more than 95% in its b (inactive, dephosphorylated) form (Cuenda et al., 1995). Interestingly, glycogen phosphorylase appears to be associated to the SR via its binding to the glycogen particles (Cuenda et al., 1994; Entman et al., 1980; Wanson et al., 1972; and Meyer et al., 1970). The binding of glycogen particles to the SR membrane may be achieved by the hydrophobic tail of the glycogen associated form of protein phosphatase 1 (Hubbard et al., 1990; Hubbard et al., 1989). There is evidence to suggest that glycogenolysis of SR glycogen results in the release of glycogen phosphorylase. Cuenda et al. (1994) showed that preparing SR from animals that were starved for 48 hours (a treatment that causes glycogen depletion) resulted in a 2 to 4 fold decrease in glycogen phosphorylase activity and content. Similarly, it was shown that amylase digestion of endogenous glycogen resulted in 95% depletion of glycogen phosphorylase (Entman et al., 1980).

Glycogen phosphorylase associated with the SR was measured three different ways: optical density of the band corresponding the 97,400 dalton molecular weight marker on a SDS-PAGE, pryidoxal 5'-phosphate (PLP) content of the SR vesicles, and glycogen phosphoylase activity associated with the SR vesicles. Three different measurements of glycogen phosphorylase were used since all three are inherently flawed. Western analysis was not practical from the SDS-PAGE because an antibody for the skeletal muscle specific isoform of glycogen phosphorylase was not available. This reduces the confidence in measuring glycogen phosphorylase using SDS-PAGE because other proteins could be present with the same molecular weight. By removing and measuring PLP, the glycogen phosphorylase content could be determined because over 95% of PLP in skeletal muscle is bound to this enzyme. However, absence of PLP does not necessarily mean that glycogen phosphorylase is not present, but glycogen phosphorylase without PLP is inactive. Finally, measurement of glycogen phosphorylase activity does not allow for quantification of enzyme content. There is no way to determine if the specific activity of the enzyme has been altered. Taken together, however, all three of the measurement techniques strongly suggest that the glycogen phosphorylase content of the SR is reduced with exercise. It seems unlikely that all three methods would falsely indicate similar changes in glycogen phosphorylase associated with the SR.

Glycogen phosphorylase activity of the SR was significantly reduced to 4.1% of control ($p \leq 0.05$). These data agree with the results from Cuenda et al. (1994) in that starvation reduced glycogen phosphorylase activity. Also, Entman et al. (1980) found that glycogen phosphorylase was reduced by 95% after α-amylase digestion of endogenous glycogen. If, in fact, glycogen phosphorylase is bound to the SR via its association with glycogen, a treatment that causes glycogen depletion should result in glycogen phosphorylase dissociation from the SR as well. Release of glycogen phosphorylase from the SR due to glycogen breakdown is compounded by the fact that glycogen phosphorylase is primarily bound to the SR in its b form (dephosphorylated, inactive), whereas muscle contraction mediates phosphorylation (activation) of this enzyme, which in turn, reduces its affinity for the SR (Cuenda et al., 1991). Pyridoxal 5-phosphate (PLP) concentration of the SR was significantly reduced to 3.3% of control ($p \leq 0.05$). These data compare well with results from glycogen phosphorylase activity. Optical density of glycogen phosphorylase determined via SDS-PAGE stained with Coomassie blue was significantly reduced to 21.2% of control ($p \leq 0.05$). These data agree with the results from Cuenda et al. (1994). Although the optical density of the band containing glycogen phosphorylase was not reduced to the same extent as PLP and glycogen phosphorylase activity, there may be a reasonable explanation. Hirata et al. (2000) has recently identified another 97,000 Dalton protein other than glycogen phosphorylase. This protein was reported to be involved in SR Ca^{2+} release, therefore, it seems likely that it may be present in the SR fraction used in the present investigation.

Decreased glycogen associated with the SR could be related to altered Ca^{2+} handling normally found in skeletal muscle fatigue, either directly or indirectly. It has been shown that SR Ca^{2+} uptake can be supported solely through the enzymatic breakdown of glycogen by glycogen phosphorylase (Nogues et al., 1996; Cuenda et al., 1993; and Montero-Lomeli et al., 1992). Also, there is evidence that suggests that glycolytic, glycogenolytic, and Ca^{2+}-accumulating enzymes are associated with the SR (Xu et al., 1995; Entman et al., 1980; and Entman et al., 1976). Decreased local ATP concentrations, due to glycogen depletion of the SR, may directly affect maximum SR Ca^{2+} uptake rates. A schematic representation of the SR membrane with glycogen, glycogen phosphorylase, and glycolytic enzymes bound in close proximity the SR Ca^{2+}-ATPase is shown in Figure 1. It has been proposed that a local increase in the ADP/ATP ratio in the triads may be responsible for decreased SR Ca^{2+} release found in fatigue. Some ATPase activity, required for SR Ca^{2+} release, utilizes ATP in the microenvironment of the triad. This ATP is not in equilibrium with the bulk cellular ATP probably because the Mg^{2+}-ATP complex is large and negatively charged, therefore, local ATP regenerating systems are necessary for the maintenance of the ADP/ATP ratio (for a review see Westerblad et al., 1998). However, experiments using caged ATP have shown no significant improvement on the slow decline of $[Ca^{2+}]_i$ late in fatigue (Allen et al., 1997). Therefore, decreased SR Ca^{2+} uptake found in fatigue may not be due to changes in [ATP] per se, but to some other mechanism.

Cuenda et al. (1991) found that glycogen phosphorylase b status of the SR affected the conformation of the SR Ca^{2+}-ATPase. These investigators found that, as glycogen phosphorylase b content increased, the binding of fluorescein isothiocyanate (FITC) to the SR proteins also increased. Since FITC binds to a specific lysine residue located in the ATP binding site of ATPase enzymes (Mitchinson et al., 1984), it was concluded that the SR Ca^{2+}-ATPase shifted towards an E2-like (ATP binding) conformation. These data suggest that increased glycogen phosphorylase concentration may allow for increased rate of ATP hydrolysis by the SR Ca^{2+}-ATPase and Ca^{2+} transport into the SR. This hypothesis suggests that the presence of glycogen and glycogen phosphorylase, and not the resulting ATP synthesis, modulates SR Ca^{2+}-ATPase function. Thus, glycogen may affect SR Ca^{2+} uptake indirectly.

Conclusion

Tetanic contractions elicited once per second for 15 minutes reduced glycogen content, glycogen phosphorylase content, and SR Ca^{2+} uptake associated with the SR. Glycogen phosphorylase activity and PLP content of the SR showed similar decreases. Loss of optical density of the band containing glycogen phosphorylase using SDS-PAGE were not as dramatic as seen with the other two measures, however, this may be explained by the presence of another protein (Hirata et al., 2000). Reduced glycogen and glycogen phosphorylase may be involved, either directly or indirectly, in a mechanism that causes decreased SR Ca^{2+} uptake normally found in fatigue.

Future directions

The following are directions for future research in the area of fatigue and SR Ca^{2+} handling:

1. Determine if digestion of glycogen associated with the SR, using α-amylase, causes decreased SR Ca^{2+} uptake.
2. Determine if glycogen synthesis in fatigued SR vesicles, with and without the presence of exogenous glycogen phosphorylase, returns SR Ca^{2+} uptake to control levels.
3. Determine if decreased glycogen, and glycogen phosphorylase associated with the SR affects Ca^{2+} release.
4. Determine if decreased glycogen, and glycogen phosphorylase alter SR Ca^{2+} release and uptake rates when they are supported solely by endogenously synthesized ATP.
5. Determine if SR vesicles incubated in α-amylase exhibit decreased FITC binding, indicating that the probability of ATP binding to the SR Ca^{2+}-ATPase is decreased as well.

CHAPTER 6: References

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