The effects of fatigue on glycogen, glycogen phosphorylase, and calcium uptake associated with the sarcoplasmic reticulum in rat skeletal muscle

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Abstract

Skeletal muscle fatigue can be defined as the inability to produce a desired amount of force. Fatigue can not only limit athletic performance and rehabilitation, but it can affect one's ability to perform every day activity as well. Despite extensive investigation of muscle fatigue, little is known about the exact mechanisms that result in decreased muscle performance. It likely involves several factors that are themselves dependent upon activation patterns and intensity. The process of excitation-contraction (EC) coupling is of particular importance with respect to regulation of force production. The release of calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR), which is stimulated by the depolarization of the sarcolemma, causes muscle contraction. The SR Ca^{2+}-adenosine triphosphatase (ATPase) drives the translocation of two Ca^{2+} ions into the SR, utilizing the energy derived from the hydrolysis of one adenosine triphosphate (ATP) molecule. The process of SR Ca^{2+} uptake causes muscle relaxation. It has been proposed that both glycogen and glycolytic enzymes are associated with the SR membrane (SR-glycogenolytic complex). Interestingly, glycogen phosphorylase, an enzyme involved in glycogen breakdown, seems to be associated with the SR-glycogenolytic complex through its binding to glycogen. The presence of the SR-glycogenolytic system may serve to locally regenerate ATP utilized by the SR Ca^{2+}-ATPase.

The purpose of the present study was to investigate the effects of prolonged muscle contraction on glycogen concentration, glycogen phosphorylase content and activity, and maximum Ca^{2+} uptake rate associated with the SR. Tetanic contractions, elicited once per second for 15 minutes, significantly reduced glycogen associated with SR to 5.1% of control from 401.17 ± 79.81 to 20.46 ± 2.16 μg/mg SR protein (£ 0.05). The optical density of glycogen phosphorylase from SDS-PAGE was significantly reduced to 21.2% of control (£ 0.05). Activity of glycogen phosphorylase, in the direction of glycogen breakdown, was significantly reduced to 4.1% of control (£0.05). Pyridoxal 5'-phosphate (PLP) concentration, a quantitative indicator of glycogen phosphorylase content, was significantly reduced to 3.3% of control (£ 0.05). Maximum SR Ca^{2+} uptake rates were significantly reduced to 80.8% of control (£ 0.05). These data suggest 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.

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CHAPTER 1: Introduction

Introduction

Prolonged muscle contraction often results in altered muscle performance. This phenomenon is referred to as muscle fatigue. Skeletal muscle fatigue manifests as a result of repetitive or sustained muscle contraction and can be characterized by a temporary reduction in the capacity to generate force. Skeletal muscle fatigue affects people in many ways. Muscle fatigue can contribute to decreased athletic performance, increased susceptibility to injury, and more seriously, it can be a debilitating symptom of certain clinical conditions like congestive heart failure. Although the changes in muscle performance are well documented in the literature, the exact mechanisms that regulate these changes in muscle performance are poorly understood.

The sarcoplasmic reticulum (SR) acts as a storage site for calcium (Ca^{2+}), as well as, controls cytoplasmic Ca^{2+} concentration, which in turn regulates the force of muscle contraction. There are cases where disease alters SR function. For example, Brody's disease is a condition where SR calcium adensoine triphosphatase (Ca^{2+}-ATPase) activity is diminished (Benders et al., 1994), which causes impaired Ca^{2+} uptake by the SR and hence, relaxation is slowed (Karpati et al., 1986). Also, some clinical conditions result in changes of phenotype of certain SR proteins. These phenotypic changes are accompanied by changes in Ca^{2+} handling properties of the SR, as well as, changes in contractile function of the muscle. For example, congestive heart failure has been shown to result in changes in SR Ca^{2+}-ATPase isoform expression (Peters et al., 1997; Simonini et al., 1996) and function (Williams and Ward, 1998; Perreault et al., 1993).

Prolonged muscle contraction results in decreased muscle glycogen concentration (Kelso et al., 1987). Glycogen appears to be specifically associated with the SR in skeletal muscle (Cuenda, 1994; Fridén, 1989; and Entman, 1976). 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). Therefore, it seems logical that prolonged muscle contraction results in decreased glycogen associated with the SR. However, the estimated amount of glycogen associated with the SR varies greatly in the literature. Cuenda et al. (1994) reported 32 μg glycogen per mg of SR membrane protein isolated from rat skeletal muscle, whereas, Entman et al. (1976) reported a range of 300 to 700 μg of glycogen per mg of SR membrane protein isolated from dog cardiac muscle.

Glycogen phosphorylase, an enzyme involved in glycogenolysis, is also associated with the SR (Wanson et al. 1972, Entman et al. 1980, and Cuenda et al. 1995). Interestingly, glycogen phosphorylase may be associated with the SR via its binding to the glycogen particles (Meyer et al. 1970 and Wanson et al. 1972). Further, glycogenolysis of SR glycogen may result in the release of glycogen phosphorylase. Cuenda et al. (1994) showed that preparing SR from rabbits 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. As for glycogen, glycogen phosphorylase associated with the SR might be expected to decline with exercise.

Many researchers have shown decreases in SR Ca^{2+} uptake as a result of prolonged muscle contraction (Ward et al., 1998; Williams et al., 1997; Williams et al., 1995; Luckin et al., 1992; Luckin et al., 1991; and Byrd et al. 1989). The process of SR Ca^{2+} uptake is mediated via SR Ca^{2+}-ATPase.

Adenosine triphosphate (ATP) is the high energy phosphate substrate needed to drive Ca^{2+} uptake into the SR. During muscle contraction, glycogenolysis of SR glycogen may provide some of the needed ATP. Also, the specific location of this system may allow for the SR Ca^{2+}-ATPase to preferentially utilize the ATP produced (Cuenda et al. 1993). It is possible that as SR glycogen is broken down, ATP is synthesized for Ca^{2+} uptake and glycogen phosphorylase is released from the SR. Also, decreased SR Ca^{2+} uptake rates found after prolonged muscle contraction may be related to this mechanism. This notion is supported by Cuenda et al. (1993). These investigators found that, as glycogen concentration was increased in their SR preparation, in the presence of added hexokinase and phosphoglucomutase (PGM), Ca^{2+} uptake rate increased as well.

Statement of Problem

Muscle glycogen is an important substrate for energy production in the cell. Glycogen breakdown provides glucose-1-phosphate, which is subsequently converted to glucose-6-phosphate and enters glycolysis. Each molecule of glucose-6-phosphate transfers 2 electrons to NAD^+, generates 2 molecules of ATP, and 2 molecules of acetyl

CoA. Acetyl CoA enters the citric acid cycle where electrons are transferred to both NAD^+ and FAD. The electrons transferred to NAD^+ and FAD are transported to the electron transport chain where the energy yield from the transfer of electrons from one molecule to another is used to create an electro-chemical gradient of H^+. This system drives the generation of ATP through controlled transport of H^+ down the electro-chemical gradient.

ATP serves several functions in the muscle cell including the maintenance of the sodium/potassium gradient across the sarcolemma, cross-bridge cycling of the contractile apparatus, Ca^{2+} uptake into the SR, and Ca^{2+} release from the SR. Because decreased muscle glycogen content is often found after prolonged muscle contraction and fatigue, it was once thought that glycogen depletion resulted in decreased ATP concentration, therefore, causing fatigue. Now, several investigations show that ATP concentration of the whole muscle changes very little with prolonged muscle contraction and fatigue (for review, see Green, 1991). There is the possibility that compartmentalization within the muscle cell allows for local changes in ATP concentration. Local changes in ATP may be responsible for some mechanisms of muscle fatigue like impaired SR Ca^{2+} release rates (Allen et al., 1997; and Körge and Campbell, 1994). It must also be considered that *in vitro* experiments involving the SR control for the extravesicular environment. Therefore, intrinsic changes of the SR membrane and/or proteins are likely responsible for altered Ca^{2+} handling and not the availability of ATP.

Glycogen is specifically associated with the SR (Cuenda, 1994; Fridén, 1989; and Entman, 1976). Glycolytic, glycogenolytic, and Ca^{2+}-accumulating enzymes also appear to be associated with the SR (Xu et al., 1995; Entman et al., 1980; and Entman et al., 1976). In addition, 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). Glycogen and glycogen phosphorylase, specifically associated with the SR, may be involved in the regulation of SR Ca^{2+} handling. Thus, the depletion of glycogen and subsequent loss of glycogen phosphorylase may be involved in a mechanism of muscle fatigue.

The specific purpose of this study was to investigate how prolonged muscle contraction affects glycogen concentration, glycogen phosphorylase content and activity, and Ca^{2+} uptake rates specifically associated with the SR. Muscle fatigue was accomplished through 15 minutes of *in situ* stimulation of one leg via the sciatic nerve, while the contralateral leg served as a control. The gastrocnemius and plantaris muscles were used for SR vesicles isolation.

Significance of Study

Muscle glycogen decreases with prolonged muscle contraction and glycogen may be depleted from certain compartments before others. Specifically, Fridén et al. (1989) found glycogen to be decreased primarily at the N_2-line of the sarcomere after prolonged muscle contraction. The N_2-line is at the lateral end of the I-band of the sarcomere, which is where the SR has been shown to be located (Sigel et al., 1969; and Pette 1975). It has yet to be established that glycogen associated with SR preparations decreases with prolonged muscle contraction. Also, it has not been established if the amount of glycogen phosphorylase (an enzyme involved in glycogen breakdown) bound to the SR is decreased with prolonged muscle contraction. Investigating the changes that occur with SR membrane bound particles, specifically enzymes and an energy substrate, may help us to better understand the changes in Ca^{2+} handling by the SR normally found after prolonged muscle contraction. Further, identification of mechanisms of fatigue may lead to clinical treatments for patients who suffer from muscle fatigue. Because muscle fatigue can be a debilitating symptom, such treatments may result in dramatic improvement in quality of life of these patients.

Specific Aims

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 a glycogen phosphorylase activity assay.

Research Hypotheses

The following are the null hypotheses tested in this investigation:

H_{O1}: Prolonged muscle contraction will have no effect on the concentration of glycogen associated with the SR.

H_{O2}: Prolonged muscle contraction will have no effect on the activity of glycogen phosphorylase associated with the SR.

H_{O3}: Prolonged muscle contraction will have no effect on the concentration of pyridoxal 5'-phosphate associated with the SR.

H_{O4}: Prolonged muscle contraction will have no effect on the optical density of the band, after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), corresponding to the molecular weight of glycogen phosphorylase using samples prepared from the SR.

Delimitations

The following delimitations were set by the investigator:

1. The treatment (*in situ* stimulation) was only administered to one leg of the animal, while the contralateral served as an unstimulated control.

2. Calcium uptake was only measured at the steepest negative slope of the free calcium versus time curve, indicating the disappearance of free calcium from the extra vesicular environment.
3. Muscle fatigue was induced by stimulation of the sciatic nerve, which can only mimic *in vivo* muscle contraction.
4. Muscle stimulation was limited to the same duration, frequency and intensity.
5. Assays were performed *in vitro*, which can only mimic the intracellular mileu.
6. The subjects were limited to only one species and gender (female Sprague-Dawley rats (200-225 g)).

Limitations and Basic Assumptions

The following are limitations, which are inherent to the experimental design, and basic assumptions made by the investigator:

1. Rats were disease and pathogen free.
2. Rats were well fed and hydrated.
3. The SR vesicles were undamaged by the isolation protocol.
4. There were no underlying factors within the muscle that would affect normal function.

CHAPTER 2: Review of the Literature

Introduction

Skeletal muscle fatigue affects people in many ways. Muscle fatigue can contribute to decreased athletic performance, increased susceptibility to injury, and more importantly, it can be a debilitating symptom of certain clinical conditions like congestive heart failure. Despite extensive investigation, mechanisms of muscle fatigue are largely unknown. The purpose of this review of the literature is to look at mechanisms of muscle fatigue as they relate to glycogen, glycogen phosphorylase, and SR function.

Excitation Contraction Coupling

Excitation contraction (EC) coupling is the process by which electrical activation of the skeletal muscle cell causes the muscle to contract. The muscle contraction process begins with the activation of the soma of an α-motor neuron. Neural depolarization is carried down the axon and delivers the electrical signal to the nerve terminal. Depolarization of the axon results in the release of acetylcholine (ACh) from the nerve terminus into the synaptic space of the neuro-muscular junction. ACh binds to receptors on the muscle membrane (sarcolemma). Binding of ACh to these receptors causes a local increase in sodium (Na^+) permeability of the sarcolemma, thereby, making the membrane potential of the sarcolemma less negative. Depolarization of the sarcolemma will occur once a threshold potential is reached via binding of ACh to the ACh receptors. Sarcolemmal depolarization is propagated the length of the cell and down through the transverse tubules (T-tubules) (reviewed in Hasson, 1994). Subsequently, this propagation affects the voltage sensitive dihydropyridine receptors (DHPR). The DHPR, in turn, causes Ca^{2+} release from the SR via the ryanodine receptor (RyR) through an unknown mechanism (Ebashi, 1991; Endo, 1977; and Ebashi, 1976). Calcium released into the cytoplasm of the cell binds to troponin (the C subunit), which relieves the inhibitory affect troponin (the I subunit) normally has on energy utilization of the contractile apparatus. The development of force is then accomplished through cross-bridge cycling of the contractile apparatus via the hydrolysis of ATP by myosin ATPase (Chalovich, 1992; Brenner, 1991; Chalovich, 1991; and Brenner, 1987). Cross-bridge cycling is the alternation of force generating and non-force generating interactions between the thick and thin filaments of the contractile apparatus. At the cessation of sarcolemmal depolarization, calcium is no longer released from the SR and is resequestered via the SR Ca^{2+}-ATPase (Martonosi, 1995). As the concentration Ca^{2+} in the cytoplasm decreases, so does the cross-bridge cycling, therefore, the muscle relaxes.

Skeletal Muscle Fatigue

Skeletal muscle fatigue is a phenomenon that can occur with repeated muscle contraction. It is characterized by a reduced shortening velocity (Edman and Mattiazzi, 1981; Crow and Kushmerick, 1983; de Haan et al., 1989; and Westerblad and Lännergren, 1994), prolongation of relaxation (Westerblad and Lännergren, 1991; Edman and Mattiazzi, 1981), and decreased maximum force generated by a muscle, due to decreases in both the Ca^{2+} sensitivity of the contractile apparatus (Westerblad and Allen, 1993; and Westerblad and Allen, 1991) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Kabbara et al., 1999; Gyorke et al., 1991; Lee et al., 1991; Allen et al., 1989). Although the exact mechanisms of muscle fatigue are unknown, it is likely that there are many different contributing factors. Further, the mechanisms of muscle fatigue may be dependent upon the stimulation intensity and frequency. For example, the mechanism of fatigue in a muscle that was stimulated at a low intensity for a prolonged period of time may be different than the mechanism for a muscle that was stimulated at a high intensity for a short period of time.

Skeletal Muscle Glycogen and Fatigue

It is well established that prolonged muscle contraction results in decreased muscle glycogen concentration (Kelso et al. 1987). Furthermore, glycogen depletion has been associated with decreased muscle performance (Stephenson et al., 1999; Chin et al., 1997; Galbo et al., 1979; Ahlborg et al., 1967; and Bergström et al., 1967). Since glycogen is an important substrate for energy production in the muscle cell, glycogen depletion may be linked to decreased force production. Although a mechanism for such a relationship has not yet been established, there is evidence that some glycogen is tightly bound to the SR (Cuenda et al., 1994; Fridén et al., 1989; and Entman et al., 1980).

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 compartmentalized distribution of glycogen within the muscle cell. These researchers suggested that glycogen is stored at 5 topographically different sites: subsarcolemmal, intermyofibrillar, para-Z-disc (in between the thin filaments at either side of the Z-disc), N_2-line (the lateral end of the I-band), and the H-zone (flanking the bare region of the myofibrillar M-band). After exercise (sixty, 8 second sprint cycling bouts), glycogen depletion was particularly noticeable at the N_2-line. 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 and the Sarcoplasmic Reticulum

Glycogen has been shown to be specifically associated with the SR using different methods (Cuenda et al., 1994; Fridén et al., 1989; and Entman et al., 1976). Both Cuenda et al. (1994) and Entman et al. (1976) used a phenol-sulfuric acid assay on isolated SR vesicles for their investigations, whereas, Fridén et al. (1989) used PA-TSC-SP staining of ultra-thin muscle sections (discussed previously). Using a sucrose density gradient isolation procedure, Entman et al. (1976) reported a range of 300 to 700 μg of glycogen per mg of SR membrane protein isolated from dog cardiac muscle. Cuenda et al. (1994), using a differential centrifugation SR isolation procedure, reported 32 μg glycogen per mg of SR membrane protein isolated from rat skeletal muscle. 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., 1993; Hubbard et al., 1989).

Sarcoplasmic Reticulum Bound Glycogen Phosphorylase

Glycogen phosphorylase, an enzyme involved in glycogenolysis, can be found in skeletal muscle in either its inactive b form (dephosphorylated) or its active a form (phosphorylated). Glycogen phosphorylase has also 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 may be associated with 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) (schematic representation of SR bound glycogen phosphorylase is shown in Figure 1). Moreover, glycogenolysis of SR glycogen may release 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 (measured using SDS-PAGE and PLP concentration). Similarly, it was shown that amylase digestion of endogenous glycogen resulted in 95% depletion of glycogen phosphorylase (measured via glycogen phosphorylase activity) (Entman et al., 1980).

Regulation of SR Bound Glycogen Phosphorylase

Glycogen phosphorylase seems to be bound to the SR in its inactive b form (Cuenda et al., 1994). Glycogen phosphorylase must be phosphorylated to its active a form for rapid glycogenolysis. Activation of glycogen phosphorylase can occur via a hormonal and/or neuronal mechanism in skeletal muscle. The hormonal pathway begins with various endocrines, epinephrine being the most potent, binding to the epinephrine receptor on the sarcolemma. This stimulates the conversion of ATP to cyclic adenosine monophosphate (cAMP) catalyzed by adenylate cyclase. cAMP then binds to the regulatory subunit of protein kinase A, causing it to be released from the enzyme. Once the regulatory subunit of protein kinase A is released, the catalytic subunit promotes the phosphorylation of phosphorylase kinase. Active phosphorylase kinase (phosphorylated) then catalyzes the reaction which phosphorylates the inactive glycogen phosphorylase b to its active form, glycogen phosphorylase a. The neuronal pathway begins with the depolarization of the cell membrane, which, in turn, causes a rapid rise in intracellular calcium. Calmodulin, a subunit of phosphorylase kinase, is also allosterically activated by calcium. Activation of calmodulin leads to the phosphorylation of phosphorylase b kinase, which results in the activation (phosphorylation) of glycogen phosphorylase (the regulatory cascade is diagrammed in Figure 2) (Hochachka and Somero, 1984). It has been shown that the regulation of this activation is affected by many factors (Parolin et al., 1999; Nogues et al., 1996; Cuenda et al., 1995; Cuenda et al., 1993; Cuenda et al., 1991; and Schwartz et al., 1976).

In 1976, Schwartz et al. investigated the effects of protein kinase and phosphorylase b kinase on SR Ca^{2+} uptake from cat slow skeletal and dog cardiac, fast skeletal, and mixed skeletal muscles. They found that phosphorylase b kinase stimulated Ca^{2+} uptake and the phosphorylation of a protein component of about 95,000 daltons. It was postulated that glycogen phosphorylase was this phosphorylated protein since it is about 94,000 daltons. The authors concluded that Ca^{2+} likely activates phosphorylase b kinase, which in turn, phosphorylates glycogen phosphorylase (b to a isoform transformation), thereby, stimulating glycogenolysis, ATP formation, and Ca^{2+} uptake.

Adenosine monophosphate (AMP) activates (Nogues et al., 1996; Cuenda et al., 1993) and caffeine inhibits SR Ca^{2+} uptake (Cuenda et al., 1993). In 1993, Cuenda et al. postulated that increases in AMP and adenosine diphosphate (ADP) concentrations in the muscle cell might activate a metabolic shuttle that produces ATP, via glycogenolysis, used to facilitate Ca^{2+} transport into the SR.

Parolin et al. (1999) investigated the time course of activation of glycogen phosphorylase over 30 second bouts of sprint exercise in humans. Subjects performed three 30 second bouts of maximal isokinetic cycling separated by 4 minutes of rest. They found that there was a rapid onset of glycogenolysis in the first 15 seconds of exercise accompanied by an increase in glycogen phosphorylase activity. By the third bout of exercise there was inhibited glycogen phosphorylase b to a conversion as well as a decrease in glycogenolysis. The authors concluded that the rapid increase in glycogen phosphorylase activity (and glycogenolysis) was due to Ca^{2+} release from the SR. As the exercise trial continued, other allosteric mediators (AMP, inosinate (IMP), and glucose-6-phosphate) decreased glycogen phosphorylase activity and oxidative phosphorylation contributed to ATP regeneration to a greater extent.

In 1995, Cuenda et al. investigated the possibility that the association of glycogen phosphorylase with less branched polysaccharides contributes to depressed activity. This was thought to be important since it has been shown that glycogen associated with the SR is less branched than glycogen purified from skeletal muscle (Wanson and Drochmans 1968). They found that the maximum reaction velocity (V_{max}) for purified glycogen phosphorylase b with α-amylose and starch (less branched polysaccharides than glycogen) was significantly lower than with glycogen. The apparent $K_{0.5\ (AMP)}$ (the estimated concentration of AMP at which the reaction rate is half its maximal value) was obtained from of glycogen phosphorylase activity data collected at varying AMP concentrations. Purified glycogen phosphorylase b associated with α-amylose and starch had a $K_{0.5\ (AMP)}$ much higher than that associated with glycogen. Also, the $K_{0.5\ (AMP)}$ for endogenous glycogen phosphorylase associated with SR membranes was much higher than for purified glycogen phosphorylase. In fact, the $K_{0.5\ (AMP)}$ for endogenous glycogen phosphorylase associated with the SR membranes was similar to the $K_{0.5\ (AMP)}$ when starch was used as a substrate instead of glycogen. The slope of the Hill plot of the dependence of glycogen phosphorylase activity on AMP concentration was 1.7-1.8. The slope of the Hill plot indicated that the binding of AMP to glycogen phosphorylase shows the characteristics of positive cooperativity. Additionally, SR membranes incubated in a medium containing the compounds needed to synthesize polysaccharide fragments caused inhibition of endogenous glycogen phosphorylase. The authors concluded that the inhibition of glycogen phosphorylase associated with the SR membrane is in part due to the more linear nature of the glycogen fragments.

Cuenda et al. (1991) investigated the interaction between glycogen phosphorylase and the SR at 25° C. They also studied this interaction with respect to the phosphorylation state of glycogen phosphorylase. The activities of both isoforms of glycogen phosphorylase (a and b) were measured in the presence of SR proteins, liposomes, and egg lecithin. Glycogen phosphorylase activity was measured in the direction of glycogen synthesis in a system containing 10 mM glucose-1-phosphate, 0.45 g/l glycogen, 0.1 mM AMP, 1 mM ethylene-glycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.15 M potassium chloride (KCl), 10 mM (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; 2-([2-hydroxy-1,1-bis-(hydroxymethyl)-ethyl]amino)ethanesulfonic acid) (TES), and 0.1 mg/ml glycogen phosphorylase. It was determined that the presence of SR proteins decreased the activity of phosphorylase b, but the liposomes and egg lecithin had no significant effect. Also, the presence of SR proteins did not affect the activity of phosphorylase a to the same extent. The effect of SR proteins on glycogen phosphorylase activity was determined in an experiment where the activity of glycogen phosphorylase was measured with varied SR membrane concentrations (0, 2, 4, 6, and 8 mg/ml SR membrane). Cuenda et al. (1995) later found that when the temperature of their assay was increased to 37˚C, the activity of glycogen phosphorylase was decreased even further. These results suggest that under physiological conditions (i.e., temperature), the activity of glycogen phosphorylase associated with the SR is decreased. For full activation of glycogen phosphorylase, it must be not only phosphorylated, but released from the SR as well.

Glycogen Phosphorylase Supported Ca^{2+} Uptake

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). There is also 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) (schematic representation of the SR-glycogenolytic complex is shown in Figure 1).

In 1976, Entman et al. studied the glycogenolytic system associated with cardiac SR. Using a sucrose density gradient isolation procedure, they found that both Ca^{2+}-accumulating and glycogenolytic enzymes sedimented in a single peak (at about 33% w/v sucrose). The contents of this peak included glycogen, adenylate cyclase, protein kinase, phosphorylase kinase, glycogen phosphoylase, and Ca^{2+}-ATPase. This observation indicated that these enzymes are associated *in vivo*. Also, this preparation exhibited a very rapid rate of glycogen phosphorylase activity as measured by a spectrophotometric assay. The authors concluded that, taken together, these data suggest that the SR contains both Ca^{2+}-sequestering and glycogenolytic enzymes *in vivo*.

Since glycogenolysis by glycogen phosphorylase results in glucose-1-phosphate production, other glycolytic enzymes must be present in order to produce the ATP needed for SR Ca^{2+} uptake. Xu et al. (1995) investigated the coupling of glycolysis and SR Ca^{2+} uptake. SR was isolated from hind-leg skeletal muscle of White New Zealand rabbits. ^{45}Ca uptake was measured through the radioactivity of SR vesicles pelleted after 15-20 minutes of incubation in a reaction mixture. When ADP (1 mM) and phosphoenol pyruvate (PEP) were added to SR vesicles, Ca^{2+}-ATPase activity was 88.9% of that when ATP (1 mM) was added. ^{45}Ca uptake supported by glycolytic substrates, cofactors, and ADP were 71 ± 22%, 28 ± 14%, 38 ± 12%, 134 ± 77%, and 107 ± 47% compared to the addition of exogenous ATP for pyruvate kinase, enolase, phosphoglyceromutase, glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase, and aldolase, respectively. At low concentrations of ADP (with PEP) or ATP (10 to 100 nM), ADP was markedly more effective than ATP in supporting ^{45}Ca uptake. The authors concluded that all of the glycolytic enzymes from aldolase onward were present in their SR isolation preparation (Figure 1). Also, endogenously formed ATP is used more effectively than exogenous ATP for ^{45}Ca transport into the SR. They proposed that ATP formed from glycolysis is directly channeled to the SR Ca^{2+}-ATPase and is not in equilibrium with bulk phase ATP.

In 1993, Cuenda et al. established that, in the presence of phosphoglucomutase (PGM), Ca^{2+} uptake increased as glycogen concentration was increased from 0 to 3.6 g/l over 10 minutes. Then, in the presence of 1.8 g/l of glycogen, Ca^{2+} uptake increased as PGM concentration was increased from 0 to 282 ug/ml over 15 minutes. Finally, the addition of 50 mM glucose almost completely eliminates Ca^{2+} uptake. This result was expected because glucose inhibits the hexokinase catalyzed synthesis of ATP from glucose-6-phosphate and ADP (Montero-Lomeli et al. 1992).

In 1996, Nogues et al. measured SR Ca^{2+} uptake rates supported by endogenously produced ATP with the addition of PGM, hexokinase, ADP, and glucose-1,6-diphosphate (physiological activator of PGM). Calcium uptake without added ATP was only measured to be about 5% of that of Ca^{2+}

uptake in the presence of ATP. However, Montero-Lomeli et al. (1992) also investigated SR Ca^{2+} uptake supported by an ATP regenerating system. This system consisted of glucose-6-phosphate, hexokinase, and ADP in a SR preparation. They reported that ATP supported Ca^{2+} uptake was only 2-3 times faster than that supported by their ATP regenerating system.

More importantly, it seems that the breakdown of glycogen, by glycogen phosphorylase, is directly linked to the sequestration of Ca^{2+} (Entman et al., 1980). This notion is known as compartmentalized microenviroments of the muscle cell. In 1980, Entman et al. showed that as the $[Ca^{2+}]_i$ decreased as a result of Ca^{2+}-sequestration, inactivation of glycogen phosphorylase followed. In addition, they found that in the presence of a Ca^{2+} ionophore (X537A), which inhibits Ca^{2+} sequestration, glycogen phosphorylase a inactivation was not observed. Also, glycogenolysis associated with the SR produces ATP nearby the Ca^{2+} ATPase, which may lead to faster and more effective relaxation of the muscle than the utilization of ATP produced elsewhere in the sarcoplasm. (Nogues et al., 1996; Xu et al. 1995).

Sarcoplasmic Reticulum Ca^{2+} Handling and Fatigue

The SR regulates intracellular calcium ($[Ca^{2+}]_i$) concentration within the skeletal muscle cell. Calcium is released from the SR through the ryanodine receptor and is sequestered via the SR Ca^{2+}-ATPase. Calcium uptake via the SR Ca^{2+}-ATPase is achieved through the hydrolysis of ATP. The energy derived from the hydrolysis of one molecule of ATP is sufficient to transport two molecules of Ca^{2+} into the SR.

Changes in the SR's ability to regulate $[Ca^{2+}]_i$ may be responsible for decreased force production normally found after prolonged muscle contraction. Many studies have shown that Ca^{2+} release rates are decreased in isolated SR preparations from exercised muscles, compared to those isolated from rested muscles (Ørtenblad et al., 2000; Ward et al., 1998; Williams et al., 1998; Belcastro et al., 1993; Favero et al., 1993; Luckin et al., 1992). This notion is also supported by studies that have shown decreased tetanic $[Ca^{2+}]_i$ in stimulated single muscle fibers isolated from amphibian (Kabbara et al., 1999; Gyorke et al., 1991; Lee et al., 1991; Allen et al., 1989) and mammalian muscle (Westerblad et al., 1993; Baker et al., 1993; Westerblad et al., 1991).

The SR Ca^{2+}-ATPase may also play a role in altered Ca^{2+} handling after prolonged muscle contraction. It has been postulated that dysfunction of this enzyme may result in either increased relaxation time, decreased Ca^{2+} availability for release, or both. The function of this enzyme can be measured using an enzyme activity assay, Ca^{2+} uptake rates, relaxation rates, or $[Ca^{2+}]_i$. Each of which will be addressed separately.

Isolated SR vesicles can be prepared using differential centrifugation or sucrose gradient techniques. Since SR proteins and membranes isolated by these techniques are resuspended in storage buffers, changes in function cannot be attributed to changes in intracellular milieu. Researchers have shown that decreased SR Ca^{2+} uptake and Ca^{2+}-ATPase activity generally accompany fatigue, either induced by prolonged (Wilson et al., 1998; Biederman et al., 1992; Luckin et al., 1991; Byrd et al., 1989a) or short term muscle contraction (Ward et al., 1998; Williams et al., 1998; Byrd et al. 1989b). These reductions in SR Ca^{2+}-ATPase activity range from about 20-60%. However, decreases in both SR Ca^{2+} uptake and Ca^{2+}-ATPase activity do not always occur together. Belcastro et al. (1993) ran groups of rats on a treadmill for 2, 15, 30, 45, and 130 minutes. They found depressed SR Ca^{2+}-ATPase activity in all exercised groups, compared to control, with 70% of the total reduction being observed after the first two minutes. However, SR Ca^{2+} uptake rates were reported to have increased in the exercise group, compared to the control. Bonner et al. (1976) also reported no changes in SR Ca^{2+}-ATPase activity isolated from untrained rats after exercise to exhaustion, compared to control samples. In fact, Bonner et al. (1976) found that SR vesicles isolated from rats trained to run on a treadmill for 30 minutes daily, five days per week had increased Ca^{2+}-ATPase activities after exercised to exhaustion. Similarly, Green et al. (1998) found that high resistance training, in human subjects, resulted in decreases in SR Ca^{2+}-ATPase activities after a prolonged exercise bout that were less pronounced than those found before training after the same exercise protocol.

Ferrington et al. (1996) investigated changes in Ca^{2+}-ATPase activity isolated from rats during post exercise recovery after two hours of treadmill running. They found that increases in Ca^{2+}-ATPase activity correlated with time. Gollnick et al. (1991) found similar results from a study done on humans. Maximum voluntary contractile strength tests (MVC) were performed and muscle biopsies were taken before, immediately after, and 30 minutes after a fatiguing bout of a one leg kicking exercise. They found depressed SR Ca^{2+}-ATPase activity and MVC immediately after the exercise, compared to before the exercise. After 30 minutes of recovery, both SR Ca^{2+}-ATPase activity and MVC increased from those measured immediately after exercise, but were still lower than the before exercise measures. In 1998, Hargreaves et al. also found increased SR Ca^{2+} uptake and ATPase activity in humans after 90 minutes of rest following three 30 second "all-out" sprint bouts on a cycle ergometer. However, Booth et al. (1997) found little recovery of SR Ca^{2+} uptake and ATPase activities in human muscle homogenates after 60 minutes rest following prolonged exercise (incremental exercise on a cycle ergometer until volitional fatigue). These investigators did find significant decreases in half relaxation time ($RT_{1/2}$) following involuntary twitch contractions, but no changes in $RT_{1/2}$ following involuntary tetanic contractions after 60 minutes rest. It is important to note, however, that $RT_{1/2}$ may not be an ideal measure of SR function.

Whole muscle homogenates can also be used to investigate SR Ca^{2+} uptake and Ca^{2+}-ATPase activity. Whole muscle homogenates are prepared simply by homogenizing the muscle sample and aliquoting directly to the desired assay. In 1999, Yashuda et al. found decreased SR Ca^{2+}-ATPase activity in whole muscle homogenates of rat soleus muscle after short (average time 2.8 minutes) and long term (average time 87.7 minutes) exercise to exhaustion. Similarly, researchers have shown decreased SR Ca^{2+} uptake and ATPase activity in humans following three 30 second "all-out" sprint bouts on a cycle ergometer (Hargreaves et al., 1998) and following prolonged exercise (Green et al., 1998; Booth et al., 1997; Green et al., 1992; and Parsons et al., 1992). However, Chin et al. (1995, and 1996) found depressed Ca^{2+} uptake rates in both isolated SR vesicles and homogenate fractions, while there were no differences in SR Ca^{2+}-ATPase activities in either of these fractions, compared to control samples.

In 2000, Ørtenblad et al. investigated changes in Ca^{2+} handling due to short-term high frequency fatigue using whole muscle homogenates. The investigators stimulated the extensor digitorum (EDL) muscles from rats *in vitro* for 4 minutes at 60 Hz, with a duty cycle of 150 milliseconds every second. Although this stimulation protocol reduced force to 14 ± 2% of prefatigue values, these researchers found no changes in either SR Ca^{2+}-ATPase or uptake rates.

Williams et al. (1997, and 1993) stimulated semitendinosus muscles of male grass frogs for 5 minutes (tetanic contractions elicited every two seconds for 100 msec at 80 Hz). Skinned fibers were isolated from both stimulated muscles and contralateral control muscles. They found decreased Ca^{2+} uptake rates of the SR estimated by the force-time integral after caffeine contractures.

Intracellular [Ca^{2+}] measurements can be used to detect transient changes in [Ca^{2+}] during muscle contraction. Measurement of $[Ca^{2+}]_i$ is done using fluorescent Ca^{2+} indicators either microinjected into muscle fibers (Westerblad et al., 1993) or used with skinned fibers (Lamb et al., (1999). SR Ca^{2+} uptake can be estimated by the rate of decrease of measured $[Ca^{2+}]_i$. Westerblad et al. (1993) found reduced rates of decrease in $[Ca^{2+}]_i$ after the last tetanus as well as elevated resting $[Ca^{2+}]_i$ after 30 minutes of rest. However, these Ca^{2+} handling properties were accompanied by dramatically reduced tetanic $[Ca^{2+}]_i$, which in turn resulted in decreased force production.

Conclusion

Several investigators have shown that fatigue results in altered Ca^{2+} handling properties of the SR. The results of these investigations have shown that the SR has diminished maximum release rates of Ca^{2+} (Ørtenblad et al., 2000; Ward et al., 1998; Williams et al., 1998; Belcastro et al., 1993; Favero et al., 1993; Luckin et al., 1992), altered Ca^{2+} transient levels (Kabbara et al., 1999; Baker et al., 1993; Westerblad et al., 1993; Gyorke et al., 1991; Lee et al., 1991; Westerblad et al., 1991; and Allen et al., 1989), and in many cases reduced Ca^{2+} uptake and ATPase activities (Ward et al., 1998; Williams et al., 1998; Wilson et al., 1998; Biederman et al., 1992; Luckin et al., 1991; Byrd et al., 1989a; and Byrd et al. 1989b). Taken together, these results suggest that the changes in SR function may be due to some intrinsic changes in the SR.

In vivo, the SR membrane is associated with glycogen as well as glycogenolytic enzymes, including glycogen phosphorylase (95% in the b form) (Cuenda et al. 1994). At physiological temperatures, the SR membrane has a greater inhibitory effect on glycogen phosphorylase in the b form than in the a form. This effect is due to both SR membrane proteins (Cuenda et al., 1992) and to its association with the less branched glycogen particles of the SR (Cuenda et al., 1995). Phosphorylation of glycogen phosphorylase (b to a conversion) activates this enzyme, which in turn causes a dramatic increase in activity and a 10-fold decrease in the apparent $K_{0.5}$ of association to the SR membrane (Cuenda, 1995). Activation of glycogen phosphorylase is regulated by several different factors (Parolin et al., 1999; Nogues et al., 1996; Cuenda et al., 1995; Cuenda et al., 1993; Cuenda et al., 1991; and Schwartz et al., 1976). Cuenda et al. (1993) showed that Ca^{2+} uptake supported by glycogen phosphorylase increased with increased glycogen concentration. Also, there seems to be a local ATP regenerating system capable of supporting Ca^{2+} uptake into the SR (Nogues et al., 1996; Xu et al., 1995; Cuenda et al., 1993; Montero-Lomeli et al., 1992; Entman et al., 1980; and Entman et al., 1976). The following questions still remain unanswered: whether prolonged muscle contraction results in decreased SR glycogen concentration, and glycogen phosphorylase associated with the SR membrane, and whether decreased SR glycogen and glycogen phosphorylase are accompanied by decreased SR Ca^{2+} uptake rates.

SR GLYCOGENOLYTIC COMPLEX

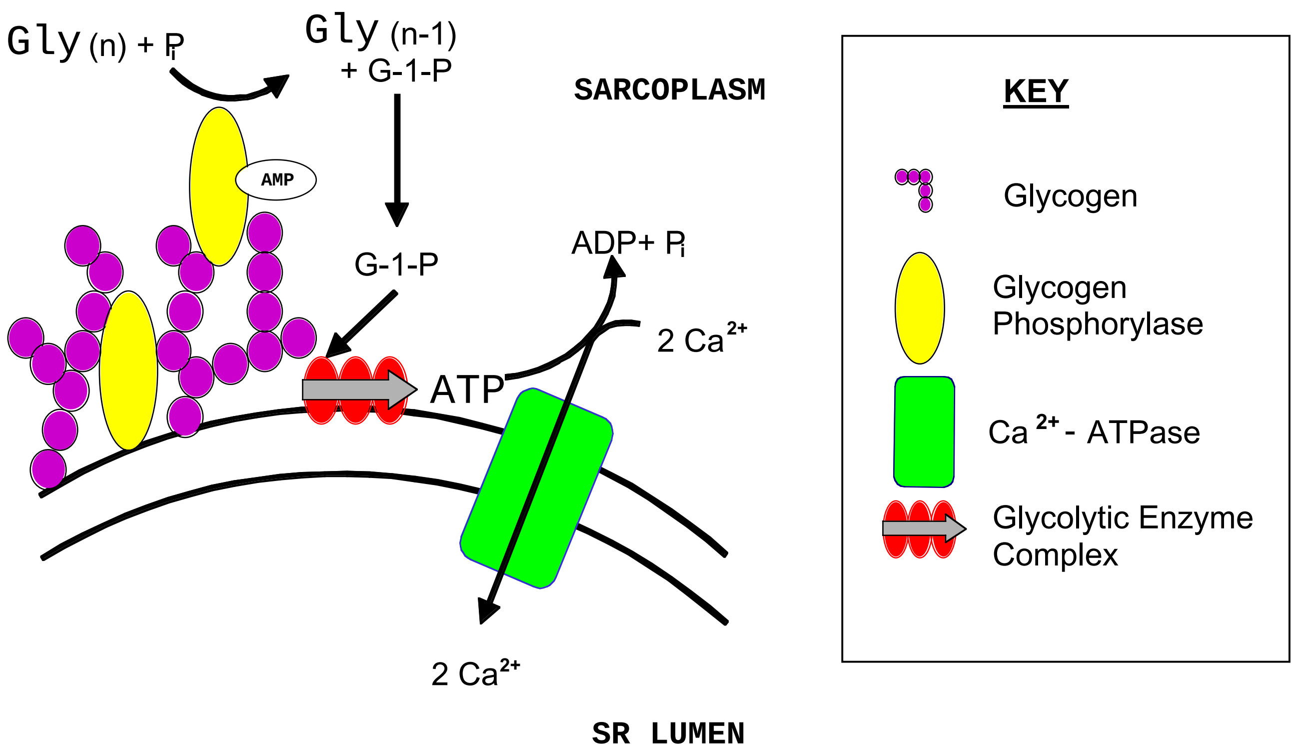

Figure 1. Schematic representation of the sarcoplasmic reticulum complex. Glycogen is anchored to the SR membrane via its binding with the SR and is associated with glycogen phosphorylase.

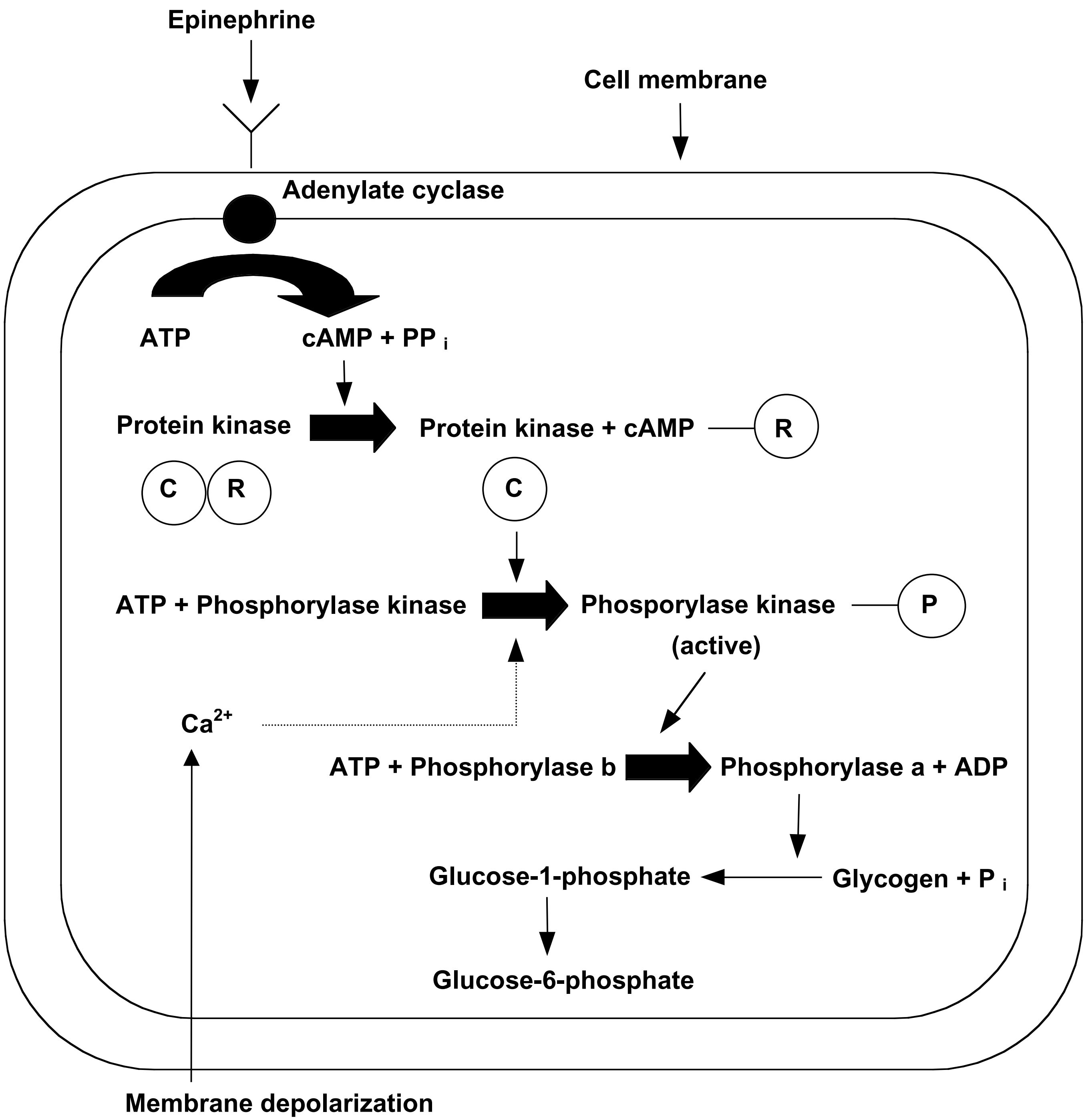

Figure 2. Schematic representation of the activation cascade of glycogen phosphorylase in a skeletal muscle cell.