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Molecular Basis for Exercise-Induced Fatigue: The Importance of Strictly Controlled Cellular Ca^{2+} Handling

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The contractile function of skeletal muscle declines during intense or prolonged physical exercise, that is, fatigue develops. Skeletal muscle fibers fatigue acutely during highly intense exercise when they have to rely on anaerobic metabolism. Early stages of fatigue involve impaired myofibrillar function, whereas decreased Ca^{2+} release from the sarcoplasmic reticulum (SR) becomes more important in later stages. SR Ca^{2+} release can also become reduced with more prolonged, lower intensity exercise, and it is then related to glycogen depletion. Increased reactive oxygen/nitrogen species can cause long-lasting impairments in SR Ca^{2+} release resulting in a prolonged force depression after exercise. In this article, we discuss molecular and cellular mechanisms of the above fatigue-induced changes, with special focus on multiple mechanisms to decrease SR Ca^{2+} release to avoid energy depletion and preserve muscle fiber integrity. We also discuss fatigue-related effects of exercise-induced Ca^{2+} fluxes over the sarcolemma and between the cytoplasm and mitochondria.

The contractile function of skeletal muscle fibers declines during intense or prolonged physical exercise, that is, fatigue develops. Within the muscle fibers, fatigue is generally related to increased energy demands, in which effective ATP resynthesis is needed to match the dramatically increased ATP consumption during contractions. In contracting muscle fibers, ATP is mainly consumed by the molecular motors—the actomyosin cross-bridges; ion pumps—the sarcoplasmic reticulum (SR) Ca^{2+}-pumps (SERCA); and, to a minor degree, the sarcolemmal Na^+-K^+-pumps. Adequate ATP delivery to these ATP-consuming proteins is essential for normal cell function and integrity, because depletion of ATP would have devastating consequences: constantly attached, noncycling cross-bridges and rigor development; insufficient SR Ca^{2+} pumping leading to an uncontrolled increase in the free cystolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$); and inadequate maintenance of Na^+ and K^+ gradients over the sarcolemma resulting in impaired action potential propagation and muscle fibers eventually becoming inexcitable. Obviously, mechanisms to prevent these catastrophic consequences of ATP depletion exist within the

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muscle fibers. These mechanisms involve, on the one hand, effective metabolic systems to resynthesize ATP and, on the other hand, a fatigue-induced decline in ATP consumption. The latter fatigue mechanisms, which inhibit contraction-dependent ATP consumption, are a major focus of this review.

The activation of skeletal muscle fibers starts at the neuromuscular junction, where an action potential arriving at the presynaptic terminal of an α-motor neuron initiates the release of acetylcholine, which binds to receptors on the muscle fiber and subsequently triggers a sarcolemmal action potential. This action potential propagates along the surface of the muscle fiber and also into the t-tubular system, in which it activates the t-tubular voltage sensors, the dihydropyridine receptors (DHPRs). DHPR then activates the SR Ca^{2+} release channels, the ryanodine receptors type 1 (RyR1), whereby Ca^{2+} is released into the cytosol and $[Ca^{2+}]_i$ increases. Ca^{2+} subsequently binds to the actin filament-regulating proteins, the troponin–tropomyosin complex. This complex undergoes conformational changes allowing the myosin heads to attach to actin, which initiates the ATP-dependent cross-bridge cycling and the muscle fiber contracts. Ca^{2+} is constantly pumped back into the SR by the ATP-consuming SERCA. $[Ca^{2+}]_i$, therefore, declines rapidly when α-motor neuron activation ceases and SR Ca^{2+} release stops, and the muscle fiber relaxes. Thus, strict control of intracellular Ca^{2+} fluxes is of fundamental importance for muscle fiber function, especially during stressful situations such as intense physical exercise.

Muscle fibers are acutely fatigued (i.e., within less than a minute to a few minutes) during intense contractile activity when the energy consumption exceeds the aerobic capacity and a large fraction of the required energy has to come from anaerobic metabolism. Accordingly, slow-twitch type 1 muscle fibers consume ATP relatively slowly, have a high aerobic capacity, and are therefore generally more fatigue resistant than fast-twitch type 2 muscle fibers (for reviews, see Allen et al. 2008; Wilson et al. 2012). Moreover, fatigue occurs more rapidly under hypoxic conditions, for example, at high altitude (Fan and Kayser 2016), when blood flow is restricted (Loenneke et al. 2014), or because of the limited O_2 diffusion in in vitro experiments performed on isolated whole muscle (Zhang et al. 2006). A fundamental effect of endurance training is increased aerobic capacity and, hence, increased fatigue resistance. It is frequently proposed that a major component behind the increased fatigue resistance with endurance training is a switch toward more slow-twitch type 1 fibers, that is, a shift from type 2 to type 1 myosin heavy chain isoforms. Although some studies have shown an increase in the proportion of type 1 fibers with endurance training (Jansson et al. 1978; Howald et al. 1985), most studies did not detect any endurance training–induced shift between the major fiber types (for reviews, see Harridge 2007; Wilson et al. 2012). Thus, a shift from type 2 to type 1 fibers rarely occurs with endurance training and the improved performance is mainly because of increased aerobic capacity within the preexisting major fiber types (Harridge 2007; Westerblad et al. 2010).

A dominating ATP-consuming process in resting muscle fibers is protein synthesis and the global protein synthesis is blunted in working muscle (Rose and Richter 2009). The decreased protein synthesis will tend to reduce ATP consumption during exercise, but the overall impact is small because the ATP consumed by cross-bridges and SERCA during contractions is several-fold higher than that of protein synthesis (Rose and Richter 2009).

Glycogen, a branched polymer containing thousands of glucose residues, serves as a readily mobilized energy store in skeletal muscle. These glycogen stores can become depleted during prolonged physical exercise (i.e., many minutes to a few hours) and there is strong correlation between glycogen depletion and fatigue-induced decline in exercise performance (Bergström et al. 1967; Hermansen et al. 1967; Ørtenblad et al. 2013).

The production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is considered to increase during physical exercise and these highly reactive molecules have been implicated in fatigue development. In addition,

force recovery after fatiguing exercise can be very slow and even take days to be completed and ROS/RNS appear to have key roles in the induction of this severely delayed recovery (Bruton et al. 2008; Cheng et al. 2016). Intriguingly, an extra intake of antioxidants has been shown to hamper beneficial effects of endurance training (Gomez-Cabrera et al. 2008; Ristow et al. 2009; Paulsen et al. 2014) and there might be common Ca^{2+}-dependent mechanisms underlying the ROS/RNS-related delayed recovery and the triggering of adaptive responses to endurance training (Place et al. 2015; Cheng et al. 2016).

In this article, we provide a concise overview of molecular and cellular aspects of exercise-induced skeletal muscle fatigue and recovery. We first discuss mechanisms behind acute fatigue with particular focus on the declining SR Ca^{2+} release in severely fatigued muscle fibers, which prevents fibers from the devastating consequences of ATP depletion. Next, we discuss the role of Ca^{2+} and ROS/RNS on the long-lasting force depression often seen after physical exercise and its relation to adaptations induced by endurance training. Finally, we discuss functional effects of exercise-induced Ca^{2+} fluxes over the sarcolemma and between the cytoplasm and mitochondria. The interest in these Ca^{2+} fluxes is increasing in view of the recent discoveries of key components: (1) the SR Ca^{2+} sensor, the stromal-interacting molecule 1 (STIM1), which activates the sarcolemmal Orai1 Ca^{2+} channel and which thereby controls store-operated Ca^{2+} entry (SOCE) (for recent reviews, see Cully and Launikonis 2013; Pan et al. 2014), and (2) the mitochondrial Ca^{2+} uniporter (MCU) and its associated regulating proteins that control mitochondrial Ca^{2+} uptake (for recent reviews, see Marchi and Pinton 2014; Williams et al. 2015; De Stefani et al. 2016).

ACUTE FATIGUE AND ITS DEPENDENCY ON ANAEROBIC METABOLISM

Fatigue develops rapidly during physical activities requiring a rate of ATP production that exceeds the aerobic capacity of the muscle fibers. Thus, this type of fatigue is closely related to the need for ATP produced by anaerobic metabolism. Experiments on single muscle fibers have revealed three components underlying the force decrease during this type of acute fatigue: decreased ability of the actomyosin cross-bridges to generate force, reduced myofibrillar Ca^{2+} sensitivity, and decreased SR Ca^{2+} release (Fig. 1) (Westerblad and Allen 1991; Allen et al. 2008). The first two of these components relate to impaired myofibrillar function and occur early during fatiguing stimulation. The third component, decreased SR Ca^{2+} release, generally becomes important in later stages of fatigue and will be discussed in more detail in the following sections.

Anaerobic metabolism leads to accumulation of lactate and hydrogen ions, mainly because of glycogen breakdown, and increased creatine and inorganic phosphate (P_i) ions, because of creatine kinase (CK)-dependent phosphocreatine breakdown (Westerblad et al. 2010). Lactate and creatine ions have no major impact on myofibrillar contractile function (Posterino et al. 2001; Murphy et al. 2004), whereas increased cytoplasmic concentrations of both H^+ (i.e., reduced pH or acidosis) and P_i have been shown to impair myofibrillar contractile function. Traditionally, "lactic acid" was considered the major cause of acute fatigue, but this viewpoint has been challenged. Many studies show a good temporal correlation between the extent of acidosis and the fatigue-induced decrease in contractile function (Cady et al. 1989; Kent-Braun 1999), but several pieces of evidence indicate that this correlation might not be causative; for instance, (1) studies have shown an early force decline during intense sustained voluntary contraction that was accompanied by alkalosis and the force recovery after fatiguing contractions can occur despite continued acidosis (Sahlin and Ren 1989; Degroot et al. 1993), (2) the force decline during fatiguing contractions was not faster when human muscle or isolated mouse flexor digitorum brevis (FDB) fibers were acidified before induction of fatigue (Fig. 2A,B) (Wilson et al. 1988; Bruton et al. 1998), and (3) at physiological temperatures, acidosis had little effect on

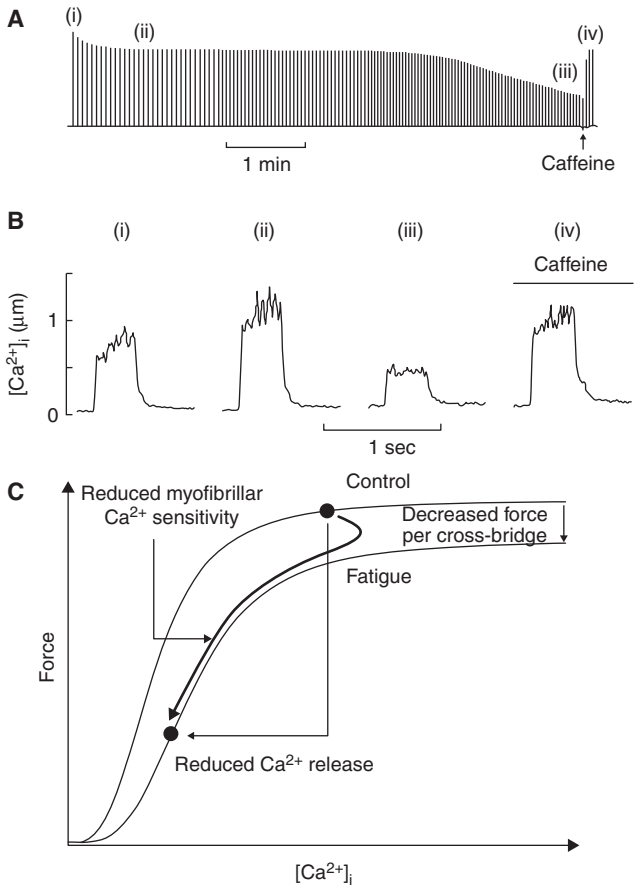

Figure 1. Three mechanisms underlying the force decrease during fatigue induced by repeated tetanic stimulation. Typical pattern of force decline (*A*), and selected $[Ca^{2+}]_i$ records (*B*) obtained during fatigue induced by repeated tetanic stimulation in mouse flexor digitorum brevis (FDB) intact single fibers. Tetanic force initially decreases while tetanic $[Ca^{2+}]_i$ increases (i to ii); hence, the initial force decline is caused by impaired myofibrillar function. The final force decrease is caused by reduced sarcoplasmic reticulum (SR) Ca^{2+} release (ii to iii). Application of caffeine facilitates SR Ca^{2+} release and the resulting increase in tetanic $[Ca^{2+}]_i$ leads to a marked force increase (iv). (*A* and *B* adapted, with permission, from Lännergren and Westerblad 1991 and Westerblad and Allen 1991, respectively.) (*C*) The relation between force and $[Ca^{2+}]_i$ during fatigue induced by repeated tetanic stimulation (thick line) plotted together with the force–$[Ca^{2+}]_i$ relationship in the unfatigued state and during fatigue (thin lines). This assessment shows that acute fatigue involves initial decreases in force per cross-bridge and myofibrillar Ca^{2+} sensitivity, which are followed by decreased SR Ca^{2+} release.

maximum force production and shortening velocity in skinned muscle fibers (Pate et al. 1995), intact mouse muscle fibers (Westerblad et al. 1997), and whole mouse muscles (Wiseman et al. 1996). Thus, increased $[P_i]$ rather than acidosis has been proposed to be the dominant cause of the declining force production during acute fatigue (Fig. 2C,D) (Dahlstedt et al. 2001; Westerblad et al. 2002; Allen et al. 2011). However, the role of acidosis in acute fatigue remains controversial and a major unresolved issue is whether the force-reducing effects of elevated $[P_i]$ in fatigue are amplified by the concomitant acidosis (Fitts 2016; Westerblad 2016).

During most types of fatigue-inducing exercise, cytoplasmic [ATP] is held relatively constant as ATP consumption is matched by ATP

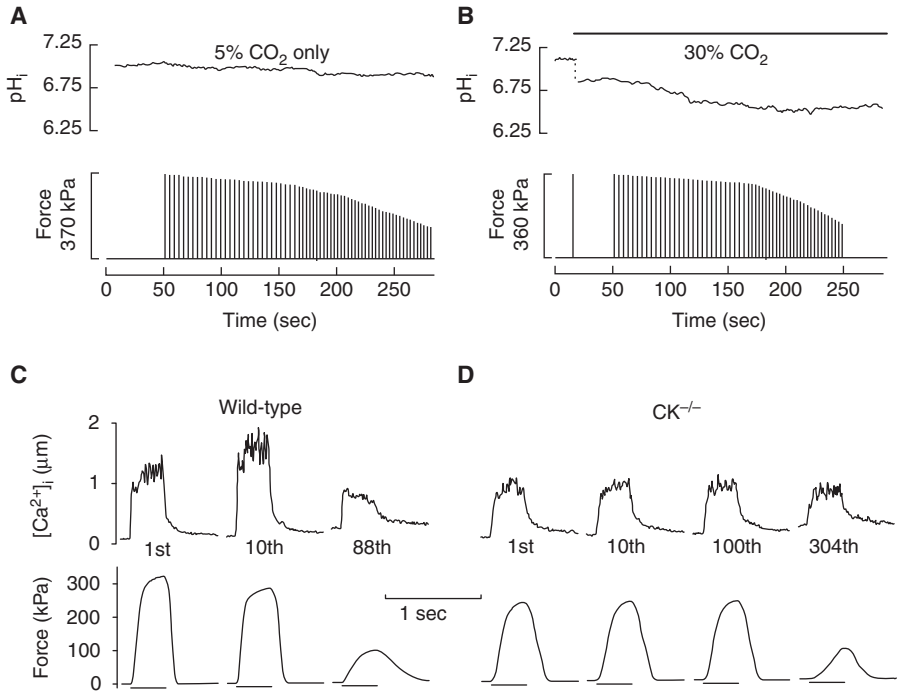

Figure 2. Acidosis has no obvious effect on fatigue development, whereas fatigue is delayed when P_i accumulation is limited by inhibition of phosphocreatine breakdown. Representative records from isolated mouse flexor digitorum brevis (FDB) intact single fibers fatigued by repeated tetanic contractions. Fibers fatigued at the same rate under normal conditions (*A*) and after being acidified by ~0.4 pH unit by bubbling the bath solution with 30% instead of 5% CO_2 (*B*). (*A* and *B* adapted, with permission, from Bruton et al. 1998.) (*C*,*D*) Fatigue developed much faster in wild-type fibers than in creatine kinase–deficient ($CK^{-/-}$) fibers, which cannot break down phosphocreatine and hence the accompanying increase in P_i is prevented. Representative records obtained in the first, tenth, and last (88th) fatiguing tetanic contraction in a wild-type fiber; the $CK^{-/-}$ fiber showed no fatigue during 100 tetanic contraction. (*C* and *D* adapted, with permission, from Dahlstedt et al. 2000.)

resynthesis by the combined action of the above discussed anaerobic sources and mitochondrial oxidative phosphorylation (Bangsbo et al. 1996; Sahlin et al. 1998; Walter et al. 1999; Westerblad et al. 2010). However, under circumstances in which very intense exercise is performed (e.g., 25 sec of maximal effort cycling), [ATP] can decrease by up to 80% in individual fast-twitch type 2 fibers, reaching values as low as 0.7–1.7 mM (Karatzaferi et al. 2001). A reduction in [ATP] is accompanied by increases in ATP breakdown products, such as ADP, AMP, and IMP. Furthermore, Mg^{2+} in the cytosol is mostly bound to ATP, and a net ATP breakdown during fatigue causes an increase in the free cytoplasmic [Mg^{2+}] ([Mg^{2+}]$_i$) (Westerblad and Allen 1992; Dahlstedt and Westerblad 2001). Thus, any or all of these metabolic changes could affect myofibrillar force generation in skeletal muscle.

During cross-bridge cycling, ATP is required to detach myosin from actin following a cross-bridge power stroke. However, a decrease in [ATP] does not have any marked effects on muscle contractility until falling below ~0.5 mM, at which point it mostly affects contractile speed and submaximal forces (Dutka and Lamb 2004; Cooke 2007). ADP acts as a weak competitor to ATP binding on the catalytic site on myosin and increased ADP will increase rather than decrease isometric force, while it slows contractions (Cooke 2007). This is supported by observations in muscle fibers from adenylate kinase–deficient mice in which [ADP] was shown to

increase to ~1.5 mM during repeated contractions, which is 20–30 times greater than typically measured during fatigue, and this was accompanied by a tendency of increased isometric force and a marked slowing of relaxation (Hancock et al. 2005). Furthermore, modeling based on the decrease in maximum shortening velocity in severely fatigued single mouse muscle fibers indicates that [ADP] may transiently increase to ~1.5 mM during tetanic contraction when the ATP-buffering phosphocreatine store has been depleted (Westerblad et al. 1998). Cytosolic concentrations of $[Mg^{2+}]_i$ that can be reached during fatigue (<3 mM) do not affect actomyosin cross-bridge properties in skeletal muscle (Swenson et al. 2014). Thus, the reduction in [ATP] and accompanying changes observed during muscle fatigue may have some relatively minor effects on the actomyosin interaction.

REDUCED SR Ca^{2+} RELEASE

As discussed above, intrinsic mechanisms must exist to prevent muscle fibers from depleting their ATP stores during high-intensity exercise. The most effective way of reducing the energy consumed by contracting muscle fibers is to inhibit SR Ca^{2+} release, which reduces the number of ATP-consuming cycling cross-bridges and lessens the demand on ATP-dependent SR Ca^{2+} reuptake by SERCA. However, reducing SR Ca^{2+} release comes with the price of declining muscle performance, that is, fatigue gets more severe. We will next discuss mechanisms by which SR Ca^{2+} might be reduced during acute fatigue.

Acidosis

Early studies showed a marked inhibition of the open probability of isolated SR Ca^{2+} release channels, RyR1, when pH was lowered to values observed in fatigued muscle fibers (Ma et al. 1988). However, later studies observed much smaller inhibitory effects of acidosis on the activation of isolated RyR1 (Laver et al. 2000). Furthermore, the normal action potential–induced SR Ca^{2+} release was little, if at all, affected by lowering pH to values observed in acute fatigue (Westerblad and Allen 1993; Lamb and Stephenson 1994; Bruton et al. 1998). Thus, acidosis is not important for the decrease in SR Ca^{2+} release in acute fatigue. In fact, recent results even show that intracellular acidosis can have a protective effect during fatigue by decreasing the permeability of chloride channels, which would help preserve muscle excitability (Pedersen et al. 2004); this will be discussed in more detail below.

Increased P_i

In addition to the effect at the myofibrillar level, an increase in $[P_i]$ has been proposed to affect SR Ca^{2+} release during acute fatigue. Experiments on intact isolated frog, mouse, and rat muscle fibers show a similar pattern of changes in tetanic $[Ca^{2+}]_i$ during fatigue induced by repeated brief tetani. An initial increase followed by a decrease and the lag between the two depends on the fatigue resistance (Allen et al. 1989; Westerblad and Allen 1991; Lunde et al. 2001). CK and elevated $[P_i]$ have a central role in this pattern of $[Ca^{2+}]_i$ changes because the initial increase is blunted and the secondary decrease is severely delayed with CK inhibition induced either pharmacologically or genetically ($CK^{-/-}$ muscles) (Dahlstedt et al. 2000; Dahlstedt and Westerblad 2001). Moreover, when the CK deficiency of $CK^{-/-}$ fibers was acutely reversed by CK injection, the normal changes in tetanic $[Ca^{2+}]_i$ during fatigue reappeared, that is, an early increase followed by a decrease (Dahlstedt et al. 2003). In the following two paragraphs, we discuss two mechanisms by which increased $[P_i]$ may decrease SR Ca^{2+} release: P_i-induced inhibition of RyR1 and Ca^{2+}-P_i precipitation within the SR.

Skinned fiber experiments have shown an inhibitory effect of increased $[P_i]$ on depolarization-induced SR Ca^{2+} release, which was attributed to impaired activation of RyR1 (Duke and Steele 2001; Steele and Duke 2003). This P_i-induced inhibitory effect was markedly larger at the $[Mg^{2+}]_i$ of fatigued (~1.6 mM) than of rested (~0.8 mM) mammalian muscle fibers (Westerblad and Allen 1992); this fits with a

relationship between decreasing tetanic $[Ca^{2+}]_i$ and increasing $[Mg^{2+}]_i$ during fatigue under normal conditions, and which is completely lost with pharmacological CK inhibition (Dahlstedt and Westerblad 2001).

The free $[Ca^{2+}]$ in the SR is assumed to be ~1 mM, and the in vitro measured Ca^{2+}-P_i solubility product is ~6 mM^2 (Fryer et al. 1995). When $[P_i]$ increases during fatigue, some P_i may enter the SR and if the Ca^{2+}-P_i solubility product is exceeded, precipitation occurs and the Ca^{2+} available for release decreases (Fryer et al. 1995). This proposed SR Ca^{2+}-P_i precipitation has not been directly shown, but indirect evidence exists both from experiments on skinned fibers with intact SR exposed to high $[P_i]$ (Fryer et al. 1995; Duke and Steele 2001; Dutka et al. 2005) and intact fibers injected with P_i (Westerblad and Allen 1996). Several fatigue experiments also support the Ca^{2+}-P_i precipitation mechanism. For instance, the $[Ca^{2+}]_i$ response to a high dose of caffeine or 4-chloro-m-cresol (compounds that directly stimulate RyR1) was reduced in fatigued mouse and toad muscle fibers, which indicates a reduction of Ca^{2+} available for rapid release (Westerblad and Allen 1991; Kabbara and Allen 1999). Accordingly, estimates of the free $[Ca^{2+}]$ in the SR using low-affinity Ca^{2+} indicators loaded into the SR show a decline during fatiguing stimulation and a subsequent recovery in both isolated toad muscle fibers and mouse muscle studied in situ (Kabbara and Allen 2001; Allen et al. 2011). The recovery of the rapidly releasable SR Ca^{2+} pool occurred in the absence of extracellular Ca^{2+}, that is, Ca^{2+} did not disappear from the cells during fatigue. On the other hand, the recovery was blocked by inhibition of mitochondrial respiration, showing that recovery of releasable SR Ca^{2+} depended on aerobic metabolism (Kabbara and Allen 1999).

Reduced ATP

RyR1 opening is strongly stimulated by ATP binding, its breakdown products ADP and AMP act as weak competitive agonists, and Mg^{2+} is a potent inhibitor (Meissner et al. 1986; Lamb and Stephenson 1994; Laver et al. 2001b; Dutka and Lamb 2004). However, rather large changes are required to induce a substantial inhibition of voltage-activated RyR1 Ca^{2+} release; for instance, cytoplasmic [ATP] has to be reduced to 0.5 mM to get an ~20% decrease in SR Ca^{2+} release, and increasing $[Mg^{2+}]_i$ to 3 mM reduces release by ~40% (Dutka and Lamb 2004). Nevertheless, the combination of changes related to declining [ATP] may decrease SR Ca^{2+} release during very intense physical activities. For instance, the transient decrease in tetanic $[Ca^{2+}]_i$ observed at the onset of high-intensity stimulation of $CK^{-/-}$ muscle fibers, where phosphocreatine cannot be used as a rapid energy buffer, was likely the result of the combined inhibitory actions of reduced [ATP] and increased $[Mg^{2+}]_i$ on RyR1 opening (Dahlstedt et al. 2000).

Impaired Action Potential Propagation

Repeated firing of action potentials during physical exercise leads to changes in intra- versus extracellular Na^+ and K^+ concentrations, which may impair sarcolemmal excitability. For each action potential, there is an influx of Na^+ during the depolarization phase and an efflux of K^+ in the subsequent repolarization phase. The resulting disturbance in Na^+ and K^+ membrane gradients is counteracted by the sarcolemmal Na^+-K^+-pumps, which actively transport Na^+ out of and K^+ in to the cell. Nevertheless, marked changes in the concentration of these ions occur during intense exercise (McKenna et al. 2008). For instance, the interstitial (extracellular) K^+ concentration can increase from ~4 mM at rest to above 10 mM during high-intensity exercise (Juel et al. 2000; Mohr et al. 2004). In rested muscle fibers, such a change in the K^+ concentration gradient over the sarcolemma results in impaired action potential propagation and, consequently, decreased force production. However, these problems are counteracted during exercise, for example, by the electrogenic effects of Na^+-K^+ pumps (McKenna et al. 2008). Moreover, Nielsen et al. (2001) showed that the impaired excitability and severely (~75%) decreased force production induced by exposing isolated rat

muscles to increased K^+ (11 mM) were almost completely reversed by acidification. This protective effect of acidosis was subsequently shown to be mediated via inhibition of sarcolemmal Cl^- (ClC-1) channels (Pedersen et al. 2004, 2005). These Cl^- channels account for 80%–90% of the passive sarcolemmal conductance, which tends to clamp the membrane at the resting potential and limit the Na^+ current-induced depolarization at the onset of action potentials. Thus, inhibition of ClC-1 channels facilitates the Na^+ current-induced depolarization and protects against action potential failure (Pedersen et al. 2016). During early stages of fatigue, ClC-1 channels are inhibited by protein kinase C–mediated phosphorylation, and by acidosis, which counteracts the reduced excitability caused by a reduced K^+ gradient over the sarcolemma (Pedersen et al. 2009a,b; de Paoli et al. 2013).

Opposite to the situation in early fatigue, the passive sarcolemmal conductance has been found to increase in late stages of fatigue in fast-twitch fibers, which promotes action potential failure (Pedersen et al. 2009b). This increased conductance was not observed in slow-twitch fibers, which indicates that it is controlled by energy metabolic factors. Moreover, experiments with pharmacological inhibitors indicate that the increased conductance is the result of parallel activation of ClC-1 channels and ATP-sensitive K^+ (K_{ATP}) channels (Pedersen et al. 2009a; de Paoli et al. 2013). The latter channels are, as the name suggests, controlled by ATP and stay in a closed state until [ATP] is decreased below ~2 mM (Spruce et al. 1985). There has been considerable interest in the possibility that K_{ATP} channels play an important role in the exercise-induced reduction in muscle force, but this remains uncertain because diverging results have been obtained both with pharmacological and genetic approaches (for review, see Flagg et al. 2010). Nevertheless, parallel opening of ClC-1 and K_{ATP} channels in late stages of fatigue constitutes a metabolically controlled mechanism by which SR Ca^{2+} release can be inhibited to prevent ATP depletion.

To sum up, several mechanisms may inhibit SR Ca^{2+} release in acute fatigue to prevent deleterious effects of ATP depletion (Fig. 3): inhibition of RyR1 caused by elevated $[P_i]$, increased $[Mg^{2+}]_i$, and/or decreased [ATP]; Ca^{2+}-P_i precipitation in the SR; decreased excitability because of a reduced K^+ gradient over the sarcolemma and opening of ClC-1 and K_{ATP} channels. How these mechanisms interact and their relative importance in different types of fatiguing exercise remain to be established. Nevertheless, the fact that several mechanisms exist shows that the ability to limit SR Ca^{2+} release in situations of severe metabolic stress is an essential property of skeletal muscle fibers.

PROLONGED EXERCISE AND GLYCOGEN DEPLETION

One of the first studies using the needle biopsy technique to obtain samples of human skeletal muscle showed a marked muscle glycogen depletion after prolonged (~2 h) cycling exercise and the time until exhaustion was correlated to the preexercise glycogen concentration (Bergström et al. 1967). Since then, many studies have confirmed that exercise capacity is severely compromised when muscle glycogen content is depleted to very low levels (Ørtenblad et al. 2013). However, the exact mechanism(s) by which glycogen depletion causes muscle fatigue remains uncertain.

Recent studies using electron microscopy imaging have shown that glycogen is preferentially located in three distinct subcellular compartments: subsarcolemmal, intermyofibrillar (i.e., between myofibrils), and intramyofibrillar (i.e., within the contracting myofibrils) (Marchand et al. 2002; Nielsen et al. 2009; Ørtenblad et al. 2013). Although the exact role of the different pools of glycogen is not fully understood, electron microscopy images show a preferential depletion of intramyofibrillar glycogen in fatigued muscle fibers from rodents (Nielsen et al. 2014, 2009) and humans (Marchand et al. 2007; Nielsen et al. 2011; Ørtenblad et al. 2011). Moreover, a correlation between reduced SR Ca^{2+} release and depletion in intramyofibrillar and, to some extent, intermyofibrillar glycogen was observed after repeated tetanic stimulation of intact single mouse muscle fibers (Nielsen

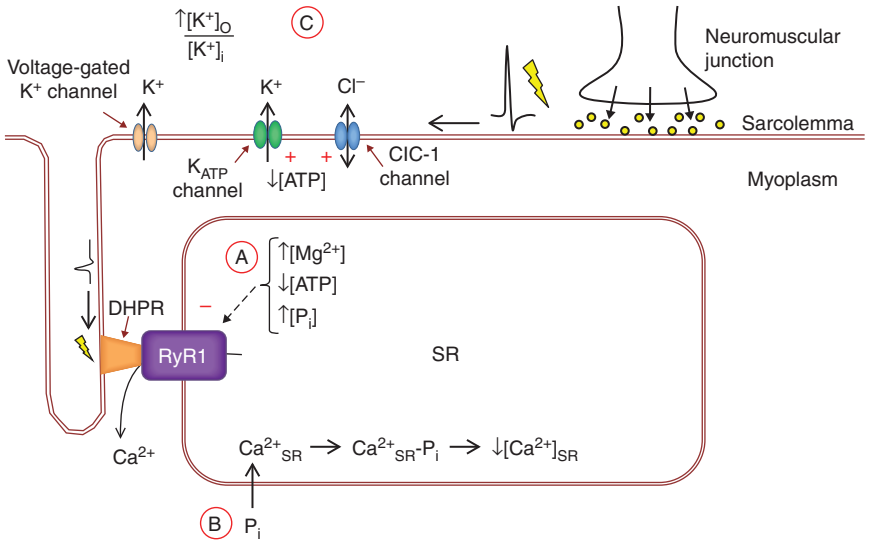

Figure 3. Cellular mechanisms of decreased sarcoplasmic reticulum (SR) Ca^{2+} release in acute fatigue. (*A*) Increases in the cytoplasmic concentration of Mg^{2+} and P_i, as well as decreased ATP, can directly inhibit action potential-DHPR-mediated RyR1 opening. (*B*) When myoplasmic $[P_i]$ increases, some P_i can enter the SR and form Ca^{2+}-P_i precipitates that reduce the free Ca^{2+} available for release ($[Ca^{2+}]_{SR}$). (*C*) Action potential propagation can be impaired by extracellular K^+ accumulation, as well as increased opening of Cl^- (ClC-1) and ATP-sensitive K^+ (K_{ATP}) channels. Red plus and minus symbols indicate excitatory or inhibitory influence on channels, respectively. DHPR, Dihydropyridine receptor; RyR1, ryanodine receptor 1; $[K^+]_o$, extracellular $[K^+]$; $[K^+]_i$, intracellular $[K^+]$.

et al. 2014). Thus, intramyofibrillar glycogen is preferentially reduced during fatigue and this is associated with decreased SR Ca^{2+} release and, hence, reduced force production. The exact mechanism(s) by which depletion of intramyofibrillar glycogen leads to decreased SR Ca^{2+} remains to be established. Future studies are also needed to elucidate links between the above-discussed mechanisms of reduced SR Ca^{2+} release in acute fatigue and localized glycogen depletion. For instance, the P_i-related impairments in myofibrillar function induced by repeated tetanic stimulation occur early during fatigue, whereas the reduced SR Ca^{2+} release attributed to Ca^{2+}-P_i precipitation comes with a delay (Dahlstedt et al. 2003; Allen et al. 2008). One possible explanation for this delay is that P_i enters the SR via small conductance chloride channels that show an increased open probability at low [ATP] (Laver et al. 2001a), which might occur in their vicinity when intramyofibrillar glycogen becomes depleted (Nielsen et al. 2014). Similar reasoning can also be applied to the delayed opening of ClC-1 and K_{ATP}, which results in increased passive sarcolemmal conductance and impaired excitability.

REACTIVE OXYGEN/NITROGEN SPECIES

ROS/RNS are molecules with unpaired valence electron(s), which makes them highly reactive (Halliwell and Gutteridge 1984). Their effects are complex and depend on several factors: the type of ROS/RNS; the magnitude, duration, and location of production; and the defense systems consisting of both endogenous and exogenous antioxidants. It is generally accepted that the production of ROS/RNS increases during most types of physical activities (Powers and Jackson 2008), although exercise-induced increases in ROS/RNS are generally difficult to measure largely because of methodological limitations (Cheng et al. 2016). For instance, fluorescent indicators used to measure cellular

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ROS/RNS show relatively small increases in fluorescence in response to repeated contractions (e.g., Reid et al. 1992; Pye et al. 2007; Sakellariou et al. 2013; Cheng et al. 2015), which makes it difficult to detect ROS/RNS changes in a quantitative and spatially/temporally efficient way. Because of these problems, methods based on high-pressure liquid chromatography (HPLC) have been recommended (Kalyanaraman et al. 2012), but such methods are impractical when following ROS/RNS over time, for example, during exercise and the subsequent recovery. Recently developed indicators based on genetically engineered fusion proteins may improve the situation, and a major attraction with these is that they can be targeted to specific organelles or proteins (Hanson et al. 2004; Gutscher et al. 2009; Meyer and Dick 2010; Pal et al. 2013).

The primary ROS in cells are the superoxide anion ($O_2^{\bullet -}$) and its downstream derivatives, such as hydrogen peroxide (H_2O_2). Mitochondria are traditionally considered to be the major site for $O_2^{\bullet -}$ production in contracting skeletal muscle and this viewpoint is, for instance, supported by recent studies employing either mitochondrial-targeted $O_2^{\bullet -}$ indicator or antioxidant (Wei et al. 2011; Cheng et al. 2015). On the other hand, other recent studies propose NADPH-oxidase 2 (NOX2) as the main ROS source in contracting skeletal muscles, because the contraction-mediated ROS increase was prevented by pharmacological inhibition or genetic knockdown of NOX2 (Michaelson et al. 2010; Pal et al. 2013; Sakellariou et al. 2013). Thus, the relative importance of different sources of $O_2^{\bullet -}$ in contracting muscle remains uncertain.

The primary RNS in cells are nitric oxide ($NO^{\bullet}$) along with its downstream derivatives, such as peroxynitrite ($ONOO^{\bullet -}$). The production of $NO^{\bullet}$ increases in muscle fibers during repeated contractions (Pye et al. 2007; Cheng et al. 2015), mainly by increased neuronal nitric oxide synthase activity (Balon and Nadler 1994; Kobzik et al. 1994; Hirschfield et al. 2000).

Clear positive effects of decreased ROS/RNS on endurance have been shown in human studies in which fatigue was produced with submaximal contractions (Reid et al. 1992, 1994; Khawli and Reid 1994; Powers et al. 2011). Conversely, the effect is small or absent with high-intensity exercise and near-maximum contractions (Fig. 4A–D) (Reid et al. 1992; Matuszczak et al. 2005; Powers et al. 2011; Cheng et al. 2015), because in this case the above-discussed "classical" fatigue mechanisms probably dominate (Allen et al. 2008). The positive effects of reducing ROS/RNS during repeated submaximal contractions fit with the fact that acute effects of exogenously applied ROS/RNS are most marked on the steep part of the force–Ca^{2+} relationship, where small changes in myofibrillar Ca^{2+} sensitivity or SR Ca^{2+} release have large effects on force production (Lamb and Westerblad 2011; Cheng et al. 2016). However, the effects of ROS/RNS are highly complex and the results of some studies even imply that reducing ROS/RNS during fatigue would impair rather than improve performance. For instance, acute exogenous application of H_2O_2 results in a transient increase in myofibrillar Ca^{2+} sensitivity (Andrade et al. 1998, 2001; Cheng et al. 2015) and some skinned fiber experiments show increased rather than decreased myofibrillar Ca^{2+} sensitivity after fatiguing contractions (Gejl et al. 2015; Watanabe et al. 2015).

After fatiguing exercise, muscle fibers frequently enter a prolonged state of severely depressed submaximal force, that is, prolonged low-frequency force depression (PLFFD) (Allen et al. 2008). At the muscle fiber level, depressed submaximal force can be caused by decreased SR Ca^{2+} release and/or reduced myofibrillar Ca^{2+} sensitivity. Acutely fatigued fast-twitch fibers of wild-type mice enter a marked PLFFD that is mainly caused by decreased SR Ca^{2+} release (Westerblad et al. 1993). On the other hand, the cause of PLFFD changes toward reduced myofibrillar Ca^{2+} sensitivity in genetically modified mouse FDB fibers overexpressing the mitochondrial matrix redox enzyme superoxide dismutase 2 (SOD2, converts $O_2^{\bullet -}$ to H_2O_2) (Bruton et al. 2008), in rat FDB fibers that endogenously express more SOD2 than mouse FDB fibers (Bruton et al. 2008), and in

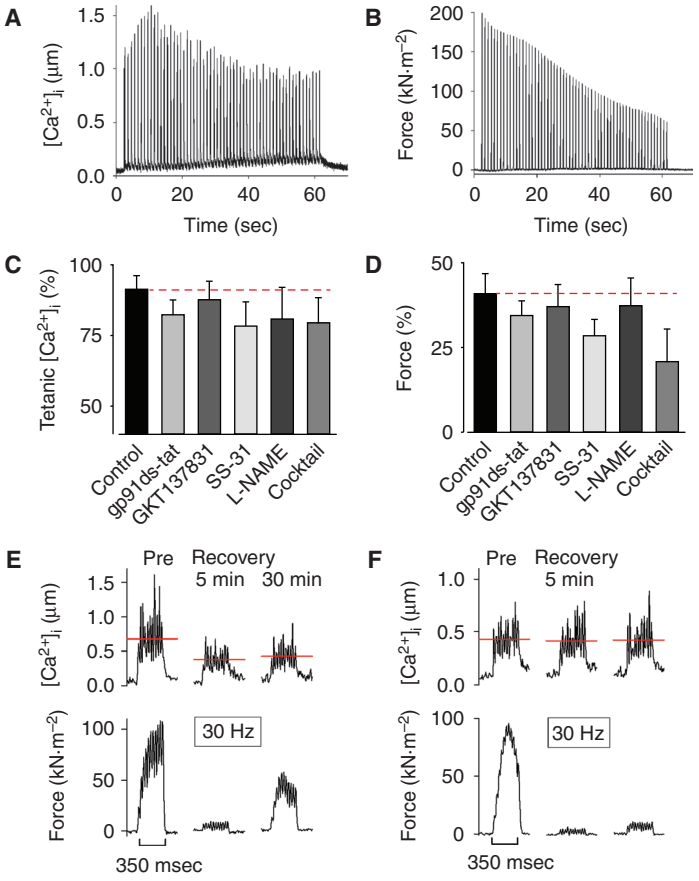

Figure 4. The decrease in tetanic $[Ca^{2+}]_i$ and force during fatigue induced by repeated tetanic contractions was not affected by the presence of reactive oxygen species (ROS)/reactive nitrogen species (RNS)-modulating compounds, whereas the delayed recovery process is ROS/RNS-sensitive. Typical fatigue profiles of $[Ca^{2+}]_i$ (*A*), and force (*B*) from an intact single flexor digitorum brevis (FDB) fiber stimulated with repeated tetanic contractions. Relative tetanic $[Ca^{2+}]_i$ (*C*), and force (*D*) at the end of fatiguing stimulation in standard Tyrode solution (control) compared with exposure to the NADPH-oxidase 2 (NOX-2) inhibitor gp91ds-tat; the NOX-4 inhibitor GKT137831; the mitochondrial-targeted antioxidant SS-31; the nitric oxide synthase (NOS) inhibitor L-NAME; and antioxidant-NOS-inhibitor cocktail. Data are mean ± SEM; dashed red lines indicate no difference from control. One-way ANOVA showed no difference between groups for either $[Ca^{2+}]_i$ or force. Representative records of $[Ca^{2+}]_i$ (*upper* row) and force (*lower* row) obtained in one control fiber (*E*) and one fiber superfused with mitochondrial-targeted antioxidant SS-31 (*F*) and stimulated at submaximal frequency (30 Hz) before (Pre) and 5 and 30 min after fatiguing stimulation. Note that the prolonged low-frequency force depression (PLFFD) was related to decreased tetanic $[Ca^{2+}]_i$ in control but not in the presence of SS-31 (indicated by red dashed lines). (Adapted, with permission, from Cheng et al. 2015.)

mouse FDB fibers treated with the mitochondria-targeted antioxidant SS-31 or the nitric oxide synthase inhibitor L-NAME (Fig. 4E,F) (Cheng et al. 2015). We recently exposed human subjects to one session of high-intensity interval training (six Wingate tests, i.e., 6 × 30 sec all-out cycling with 4 min rest in between) (Place et al. 2015). This exhaustive exercise caused a marked PLFFD accompanied by an unexpected RyR1 fragmentation in vastus lateralis muscles of recreationally active subjects. A similar PLFFD was induced in muscles of elite endurance athletes, but the RyR1 remained intact. The elite endurance athletes had higher levels

of the antioxidant enzymes SOD2 and catalase in their muscles than recreationally active subjects. Furthermore, RyR1 fragmentation was induced by high-intensity stimulation of isolated mouse muscles and this fragmentation was blocked by the antioxidant *N*-acetyl cysteine (Place et al. 2015). To sum up, accumulation of $O_2^{\bullet -}$, or $ONOO^{\bullet -}$, preferentially impairs SR Ca^{2+} release, likely via redox modifications of RyR1 (Bellinger et al. 2008a,b; Andersson et al. 2011; Lanner et al. 2012). On the other hand, decreased myofibrillar Ca^{2+} sensitivity is the major problem when $O_2^{\bullet -}$ is more effectively metabolized to H_2O_2, or its downstream products. Thus, antioxidants do not prevent PLFFD, but they can change the underlying mechanism from impaired SR Ca^{2+} release to reduced myofibrillar Ca^{2+} sensitivity (Cheng et al. 2016).

An increase in baseline $[Ca^{2+}]_i$ can stimulate mitochondrial biogenesis and hence lead to improved muscle endurance (Wright et al. 2007; Bruton et al. 2010). As described above, PLFFD in muscle of wild-type mice and recreationally active humans was accompanied by RyR1 modification, which can elevate baseline $[Ca^{2+}]_i$, and thereby trigger mitochondrial biogenesis and other beneficial adaptations (Bruton et al. 2010; Place et al. 2015). Thus, ROS/RNS-induced modifications in RyR1 structure and function offer a mechanism as to why treatment with antioxidants hamper the beneficial effects of endurance training (Gomez-Cabrera et al. 2008; Ristow et al. 2009; Paulsen et al. 2014; Merry and Ristow 2016). However, RyR1 modification can be a double-edged sword and severe modifications, for example, a major RyR1 depletion of the associated stabilizing protein FKBP12, have been linked to muscle weakness after strenuous contractile activity (Aydin et al. 2008; Bellinger et al. 2008b), during aging (Andersson et al. 2011), or in various pathological conditions (Bellinger et al. 2008a; Waning et al. 2015).

Ca^{2+} FLUXES OVER THE SARCOLEMMA

The contractile machinery in skeletal muscle fibers is activated by Ca^{2+} ions being released from the SR. The absolute majority of the Ca^{2+} ions being released are subsequently pumped back into the SR by the SERCA (Balnave and Allen 1998; Cully and Launikonis 2013). However, a small amount of the released Ca^{2+} will leave the cell, mainly via the Na^+-Ca^{2+} exchanger and plasma membrane Ca^{2+}-ATPase (Hidalgo et al. 1986; Balnave and Allen 1998). This efflux of Ca^{2+} has to be countered by a Ca^{2+} influx to avoid Ca^{2+} depletion during physical activities. Accordingly, the rate of Ca^{2+} influx is increased during prolonged contractile activities and, if anything, the total muscle Ca^{2+} content increases (Everts et al. 1993; Gissel and Clausen 1999).

During contractions, SR Ca^{2+} release is initiated by action potential activation of DHPR, which in turn activates RyR1. DHPR, also called $Ca_v1.1$, is a voltage-dependent L-type Ca^{2+} channel that, because of its slow Ca^{2+} conductance, mainly acts as voltage sensors in skeletal muscle (Lamb 2000). Nevertheless, Ca^{2+} may enter muscle fibers via action potential activation of the DHPRs (Lee et al. 2015; Robin and Allard 2015).

Ca^{2+} can also enter muscle fibers via SOCE (Cully and Launikonis 2013; Pan et al. 2014). In this case, decreased SR $[Ca^{2+}]$ stimulates the intra-SR Ca^{2+} sensor, STIM1, which activates the sarcolemmal Orai1 Ca^{2+} channel mainly located in the t-tubular membrane. A recent study supports an important role of SOCE during fatiguing contractions: knockdown of STIM1 or expression of a dominant-negative Orai1 mutation in mouse FDB fibers accelerated the decline in $[Ca^{2+}]_i$ during repeated tetanic stimulation; dominant-negative Orai1 mice displayed impaired performance in in vivo fatigue tests and muscles isolated from these mice fatigued more rapidly during repeated contractions (Wei-Lapierre et al. 2013). However, enhanced Ca^{2+} influx via SOCE induced by overexpressing STIM1 in mouse skeletal muscle resulted in severe pathological changes in muscle fibers resembling those in muscle dystrophy (Goonasekera et al. 2014). Thus, a fine-tuned balance between Ca^{2+} influx and efflux is essential for overall muscle function and integrity. For instance, increased efflux of Ca^{2+} during

fatiguing contractions must be balanced by a similarly sized Ca^{2+} influx to keep the SR Ca^{2+} store intact. Accordingly, the decrease in tetanic $[Ca^{2+}]_i$ observed during late stages of fatigue is caused by impaired SR Ca^{2+} release rather than cellular Ca^{2+} depletion (see Fig. 1A,B).

MITOCHONDRIAL Ca^{2+} UPTAKE

Mitochondria are located adjacent to the SR, and are tethered together with small (10 nm) linkages between the outer mitochondrial membrane and the SR (Boncompagni et al. 2009). The close proximity between mitochondria and SR implies important interactions between the two organelles. Ca^{2+} readily passes into the mitochondrial intermembrane space via the outer membrane voltage-dependent anion channel (VDAC) (Ben-Hail et al. 2014). Subsequently, Ca^{2+} can enter the mitochondrial matrix mainly via the mitochondrial calcium uniporter (MCU) (Kirichok et al. 2004; Baughman et al. 2011; De Stefani et al. 2011). Finally, Ca^{2+} is extruded from the mitochondrial matrix mainly by the mitochondrial Na^+-Ca^{2+} exchanger (NCLX) (Palty et al. 2010). Thus, the free $[Ca^{2+}]$ in the mitochondrial matrix ($[Ca^{2+}]_{mito}$) is set by the dynamic balance between MCU Ca^{2+} entry and NCLX Ca^{2+} extrusion (Williams et al. 2015). To avoid deleterious effects of mitochondrial Ca^{2+} overload (Duchen 2000; Brookes et al. 2004), $[Ca^{2+}]_{mito}$ must be tightly controlled and not simply reflect $[Ca^{2+}]_i$, which can increase ~100-fold when skeletal muscle fibers contract. Accordingly, MCU is regulated by several inner mitochondrial membrane proteins, which either stimulate or inhibit Ca^{2+} entry (Williams et al. 2015). The existence of an intricate regulation of $[Ca^{2+}]_{mito}$ is illustrated by studies with repeated tetanic contractions of mouse wild-type skeletal muscle fibers, where the repeated large increases in $[Ca^{2+}]_i$ during contractions were accompanied by $[Ca^{2+}]_{mito}$ remaining virtually constant (Lännergren et al. 2001; Bruton et al. 2003b; Aydin et al. 2009), increasing substantially in mitochondria close to the surface (Bruton et al. 2003a), or increasing substantially and globally (Rossi et al. 2011; Ainbinder et al. 2015).

Increased $[Ca^{2+}]_{mito}$ is a known stimulator of mitochondrial respiration and ATP production (Brookes et al. 2004; Glancy et al. 2013). Accordingly, impaired performance in a treadmill running endurance test was observed in MCU-deficient mice (Pan et al. 2014). On the other hand, excessive mitochondrial matrix Ca^{2+} uptake induces apoptotic signaling and can even result in cell death (Duchen 2000; Brookes et al. 2004). This can be illustrated by the mouse myopathy model with skeletal muscle-specific disruption of mitochondrial transcription factor A (*Tfam*). In contrast to their controls, muscle fibers of the *Tfam* knockout mice showed a large increase in $[Ca^{2+}]_{mito}$ during repeated contractions and this increase was partially inhibited by preexposure to cyclosporine A (Aydin et al. 2009). The muscle-specific *Tfam* knockout mice develop a progressive muscle weakness because of decreased SR Ca^{2+} stores and they die when they are ~4 months old; treatment with cyclosporine A counteracted the development of muscle weakness by improving SR Ca^{2+} handling, and all treated mice were still alive at 4 months of age (Gineste et al. 2015). To sum up: (1) there are intricate, functionally important, and not yet fully understood interactions between SR and mitochondria, which involve bidirectional Ca^{2+}-dependent signaling; and (2) $[Ca^{2+}]_{mito}$ must be tightly controlled to maintain the fine balance between beneficial and deleterious Ca^{2+} effects.

There are still uncertainties whether mitochondrial Ca^{2+} uptake represents a substantial Ca^{2+} buffer in cells and in this way significantly influences transient changes in $[Ca^{2+}]_i$ (Williams et al. 2013). Quantitative analyses and simulations indicate that mitochondrial Ca^{2+} uptake has no or very limited impact on $[Ca^{2+}]_i$ transients under normal physiological conditions (Baylor and Hollingworth 2007; Williams et al. 2013), and this agrees with results obtained with repeated contractions of mouse soleus muscle fibers (Bruton et al. 2003a). Conversely, mitochondrial Ca^{2+} uptake appeared to affect tetanic $[Ca^{2+}]_i$ in muscle fibers of *Xenopus* frogs (Lännergren et al. 2001), and the decreased mitochondrial Ca^{2+} uptake during contractions was accompanied by in-

Table 1. Examples of exercises in which different fatigue mechanisms might limit performance

Mechanism underlying impaired performance	Type of exercise
Decreased myofibrillar force-generating capacity	Three to five repetition maximum weight lifts
Decreased SR Ca^{2+} release caused by increased $[P_i]$	400–800 m running; 100–200 m swimming
Decreased SR Ca^{2+} release caused by reduced [ATP] and increased $[Mg^{2+}]_i$	All-out Wingate cycling
Impaired action potential propagation	Sustained maximum voluntary contraction; neuromuscular electrical stimulation (NMES)
Muscle glycogen depletion	Half-marathon and marathon running; 50 km cross-country skiing
Increases in ROS/RNS	Recovery after high-intensity or prolonged exhaustive exercises

creased $[Ca^{2+}]_i$ transients in mouse muscle fibers exposed to short-term knockdown of mitofusin-2, which participates in mitochondrial fusion (Ainbinder et al. 2015).

CONCLUDING REMARKS

Within skeletal muscle fibers, acute fatigue develops when fibers depend on anaerobic metabolism. Early stages of fatigue involve impairments in myofibrillar functions: decreased cross-bridge force-generating capacity and reduced Ca^{2+} sensitivity. Impaired SR Ca^{2+} release becomes more important in later stages of fatigue and protects against highly deleterious consequences of energy depletion. Several mechanisms can contribute to the decreased SR Ca^{2+} release induced by high-intensity fatiguing exer-

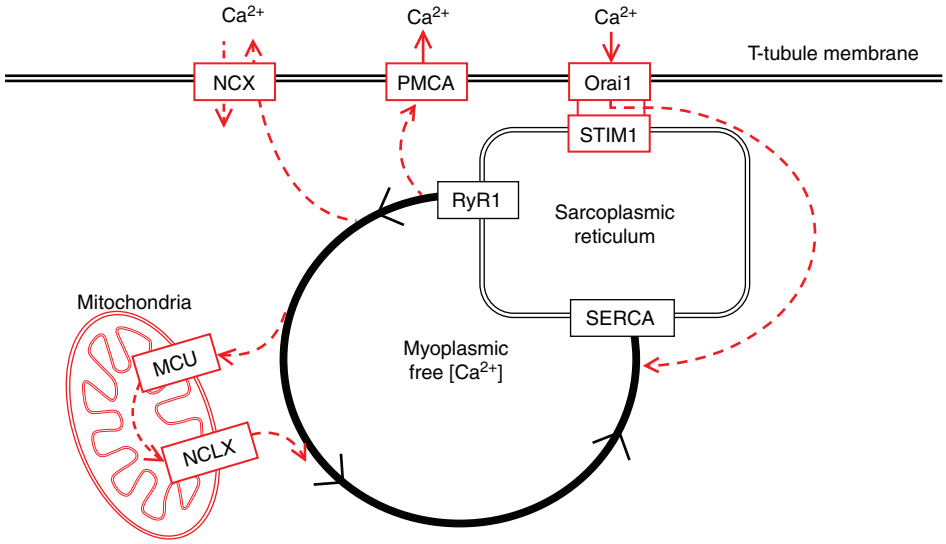

Figure 5. Ca^{2+} fluxes during contractions of skeletal muscle fibers. The contractile machinery of muscle fibers is triggered by Ca^{2+} released from the sarcoplasmic reticulum (SR) via the ryanodine receptor 1 (RyR1) Ca^{2+} channels. The absolute majority of Ca^{2+} ions are actively pumped back into SR by the SR Ca^{2+} pumps (SERCA). Few Ca^{2+} ions are extruded from the cell via the plasma membrane Ca^{2+} (PMCA) pumps or Na^+-Ca^{2+} exchangers (NCX); the Ca^{2+} extrusion is balanced mainly by store-operated Ca^{2+} entry, which involves the SR Ca^{2+} sensor, the stromal-interacting molecule 1 (STIM1) that activates the sarcolemmal Orai1 Ca^{2+} channels. Some Ca^{2+} ions may enter the mitochondrial matrix mainly via the tightly controlled mitochondrial Ca^{2+} uniporter (MCU) and these are subsequently returned to the myoplasm via mitochondrial Na^+-Ca^{2+} exchangers (NCLX).

cise (see Fig. 3). The reduction in SR Ca^{2+} release is related to glycogen depletion with more prolonged lower intensity exercise. Finally, increased ROS/RNS can cause prolonged impairments in SR Ca^{2+} release and/or myofibrillar Ca^{2+} sensitivity and, hence, prolonged force depression, which might protect muscle fiber integrity after extensive exercise. Increased ROS/RNS can also trigger beneficial adaptations to endurance exercise. Table 1 presents examples of exercises in which the above-described fatigue mechanisms might limit the performance.

Figure 5 summarizes Ca^{2+} fluxes activated during repeated contractions. The absolute majority of Ca^{2+}-ions released from the SR via RyR1 are actively pumped back into the SR by the SERCA. In addition, small amounts of Ca^{2+} ions leave the cell or enter the mitochondrial matrix. Although the latter fluxes are quantitatively small, they may affect fatigue development: impaired reuptake of extruded Ca^{2+} because of defective SOCE has been shown to accelerate fatigue development; modestly increased $[Ca^{2+}]_{mito}$ speeds up mitochondrial respiration, whereas an excessive increase may trigger apoptotic signaling and even induce cell death.

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