Invited Review

HIGHLIGHTED TOPIC | *Fatigue Mechanisms Determining Exercise Performance*

Impaired calcium release during fatigue

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Allen DG, Lamb GD, Westerblad H. Impaired calcium release during fatigue. *J Appl Physiol* 104: 296–305, 2008. First published October 25, 2007; doi:10.1152/japplphysiol.00908.2007.—Impaired calcium release from the sarcoplasmic reticulum (SR) has been identified as a contributor to fatigue in isolated skeletal muscle fibers. The functional importance of this phenomenon can be quantified by the use of agents, such as caffeine, which can increase SR Ca^{2+} release during fatigue. A number of possible mechanisms for impaired calcium release have been proposed. These include reduction in the amplitude of the action potential, potentially caused by extracellular K^+ accumulation, which may reduce voltage sensor activation but is counteracted by a number of mechanisms in intact animals. Reduced effectiveness of SR Ca^{2+} channel opening is caused by the fall in intracellular ATP and the rise in Mg^{2+} concentrations that occur during fatigue. Reduced Ca^{2+} available for release within the SR can occur if inorganic phosphate enters the SR and precipitates with Ca^{2+}. Further progress requires the development of methods that can identify impaired SR Ca^{2+} release in intact, blood-perfused muscles and that can distinguish between the various mechanisms proposed.

calcium channel; isolated skeletal muscle fibers

THE DECLINE IN MUSCLE PERFORMANCE with intense activity is one of the most widely recognized properties of muscle; however, the mechanisms involved are complex, poorly defined, and controversial. This brief review focuses on one aspect of muscle fatigue, the impairment of calcium release from the sarcoplasmic reticulum (SR). This idea was first proposed in 1963 by Eberstein and Sandow (49) on the basis that both caffeine and high extracellular K^+, agents capable of facilitating Ca^{2+} release from the SR, could partially overcome the reduced force of fatigue. This idea was confirmed and extended when it became possible to measure myoplasmic free calcium concentration ($[Ca^{2+}]_{myo}$) in single fibers that were stimulated to fatigue. In 1989, Allen et al. (2) used aequorin to measure $[Ca^{2+}]_{myo}$ in frog fibers and were the first to show that the tetanic $[Ca^{2+}]_{myo}$ first increased and then decreased during repeated tetani leading to fatigue (see Fig. 1*B*). This pattern has proved to be widespread, having been observed also in fast-twitch mouse and rat fibers. Data from slow-twitch fibers are more sparse, partly because they are so fatigue resistant that it is difficult to study fatigue mechanisms. Mouse soleus fibers showed the pattern described above with an early increase in tetanic $[Ca^{2+}]_{myo}$ followed by a decrease, except that these changes occurred over 1,000 tetani instead of ~100 tetani in fast-twitch fibers (21). Rat soleus fibers, on the other hand, did not display the early increase in tetanic $[Ca^{2+}]_{myo}$ during fatigue induced by

repeated tetani (85). The fact that slow-twitch fibers can better maintain high tetanic $[Ca^{2+}]_{myo}$ during fatigue is related to their high aerobic capacity, because tetanic $[Ca^{2+}]_{myo}$ was rapidly decreased when mouse soleus fibers were fatigued in the presence of cyanide, which inhibits mitochondrial respiration (129).

The functional importance of this decline in SR Ca^{2+} release in fatigued muscle has been established in several ways. The application of caffeine to a fatigued muscle overcomes much of the late decline of force (phase 3 in Fig. 1*A*) and is associated with a substantial increase in the tetanic $[Ca^{2+}]_{myo}$ signal [Fig. 1*B(iv)*]. This provides strong evidence that the impairment of SR Ca^{2+} release is the cause of the final phase of fatigue. An alternative approach is plot the relation between tetanic $[Ca^{2+}]_{myo}$ and force in a single fiber before fatigue by varying the stimulus frequency (left curve in Fig. 1*C*). The relation between tetanic $[Ca^{2+}]_{myo}$ and force can also be plotted in the final phase of fatigue (right curve in Fig. 1*C*). The attraction of this approach is that from these two curves it is possible to determine the contributions to the reduced force during fatigue of *1*) decline in tetanic $[Ca^{2+}]_{myo}$, *2*) the decline in Ca^{2+} sensitivity of the myofibrillar proteins, and *3*) the decline in maximum Ca^{2+}-activated force (121). This approach shows that phase 1 is caused by a decline in maximum Ca^{2+}-activated force, whereas phase 3 has contributions from both reduced tetanic $[Ca^{2+}]_{myo}$ and reduced Ca^{2+} sensitivity. The present review is concerned with decline in tetanic $[Ca^{2+}]_{myo}$, which is principally caused by impaired SR Ca^{2+} release. The reduced Ca^{2+} sensitivity and maximum Ca^{2+}-activated force under the conditions of Fig. 1
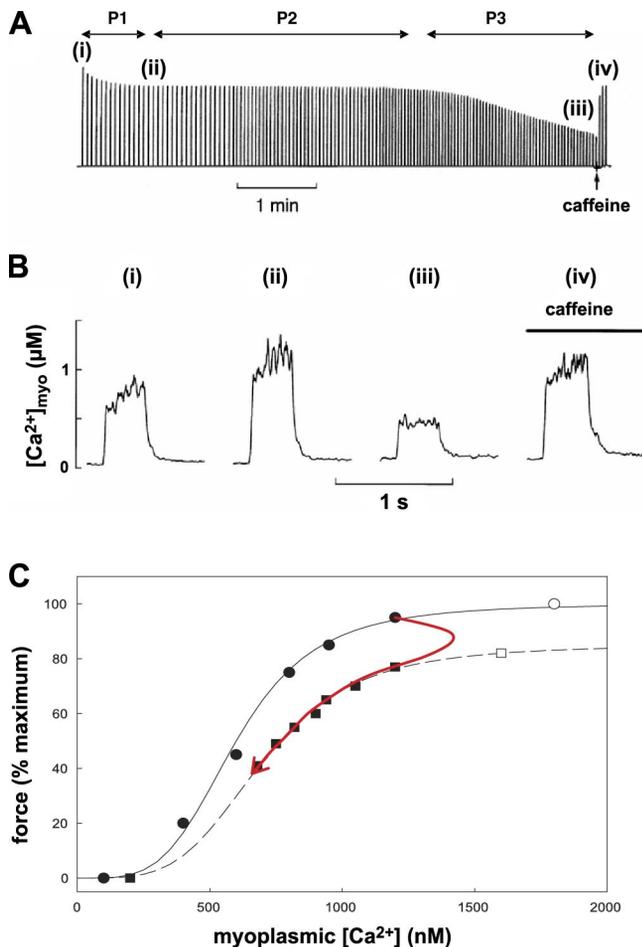
Fig. 1. Analyzing the contribution of impaired Ca^{2+} release to muscle fatigue. *A*: force record of repeated, short tetani continued until force had declined 40%. P1 indicates the first phase in which force shows a small and rapid decline. P2 shows a stable phase in which force is little changed. P3 shows the final phase in which the rate of decline of force accelerates. *i–iv* indicate representative tetani whose tetanic $[Ca^{2+}]_{myo}$ are shown in *B*. The final 3 tetani in *A* show the effect of rapid application of 10 mM caffeine. Note that force rapidly recovers to the level of P2. *B*: tetanic $[Ca^{2+}]_{myo}$ from tetani illustrated in *A*. Note the fall in tetanic $[Ca^{2+}]_{myo}$ from *ii* to *iii*, which is associated with the reduced force of P3. The causal importance of the fall in tetanic $[Ca^{2+}]_{myo}$ is indicated by the recovery of force when caffeine increases tetanic $[Ca^{2+}]_{myo}$. *A* and *B* are modified from Ref. 3. *C*: graphical analysis of the contributions of *i*) reduced tetanic $[Ca^{2+}]_{myo}$, *ii*) reduced Ca^{2+}-sensitivity, and *iii*) reduced maximum Ca^{2+}-activated force. The *left* plot (●) shows data from unfatigued muscle in which tetanic $[Ca^{2+}]_{myo}$ was altered by changes in stimulus frequency and application of 10 mM caffeine (○). The dashed line and ■ shows force and tetanic $[Ca^{2+}]_{myo}$ from P3 of the experiment in *A* and *B*. □ shows the force and tetanic $[Ca^{2+}]_{myo}$ from the final application of caffeine. The red line shows the trajectory of force and $[Ca^{2+}]_{myo}$ during the fatigue run. Modified from Ref. 121.

(room temperature) are thought to result from the combined effects of inorganic phosphate (P_i) accumulation and acidosis (1).

It is generally accepted that impaired SR Ca^{2+} release occurs in fatigued muscles and makes a substantial and quantifiable contribution to the decline in force. However, multiple mechanisms capable of reducing SR Ca^{2+} release have been identified and there is little agreement on which are important during muscle fatigue. In this review we consider each of the main contenders that are also illustrated in Fig. 2.

ACTION POTENTIAL DECLINE AND VOLTAGE-SENSOR ACTIVATION OF RYRS

Muscle contraction depends on action potentials (APs) triggering adequate release of Ca^{2+} from the SR. Each AP generated at the neuromuscular junction propagates rapidly along the surface membrane of the muscle fiber (AP SL in Fig. 2) and into the transverse tubular system (AP TT in Fig. 2), where it is detected by voltage-sensor molecules (the dihydropyridine receptors, VS/DHPRs in Fig. 2), which in turn open the ryanodine receptor-Ca^{2+} release channels in the adjacent SR (SR Ca^{2+} RC/RYR1 in Fig. 2; Ref. 87). The generation and propagation of APs requires the activation of voltage-dependent Na^+ channels (Nav1.4; Ref. 70). Each AP involves the initial influx of a very small amount of Na^+ into the muscle fiber, and the subsequent repolarization involves some K^+ efflux (32, 105). Repeated stimulation at high frequency thus may lead to increased intracellular $[Na^+]$, reducing the electrochemical gradient for Na^+ influx, to increased extracellular $[K^+]$ and to decreased intracellular $[K^+]$. Of these changes, the increase in extracellular $[K^+]$ near the muscle fiber, which can rise from 4 to ~10 mM or more (32, 105), is the most significant because this substantially reduces the equilibrium potential for K^+, which in turn will reduce the resting membrane potential of the fiber. If the membrane potential were to become less negative than approximately −55 mV, the Na^+ channels would fail to recover from inactivation, stopping all AP activity and muscle contraction (53). However, such complete AP failure probably does not occur during normal exercise in vivo. A number of studies have recorded the "M wave" from muscles in exercising humans. This is an extracellular recording of the compound electrical signal as an evoked AP traverses along the muscle fibers. In a number of studies, the M
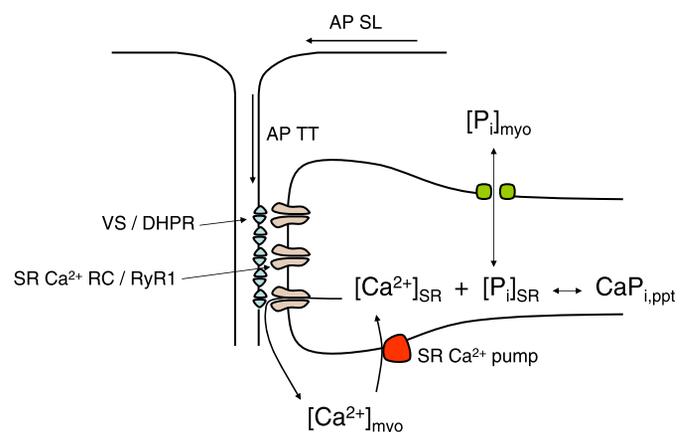

Fig. 2. Structures involved in excitation-contraction coupling in skeletal muscle. Diagram shows representative sections of the surface membrane, T tubule, and sarcoplasmic reticulum. APs generated at the neuromuscular junction conduct along the sarcolemma (AP SL) and are then actively conducted down the T tubule (AP TT). Modified Ca^{2+} channels in the T tubule, also known as voltage sensors or dihydropyridine receptors (VS/DHPR) interact with the SR Ca^{2+}-release channels (SR Ca^{2+} RC), also known as ryanodine receptors (RyR1 isoform in skeletal muscle) and cause release of Ca^{2+}, which elevates the myoplasmic calcium ($[Ca^{2+}]_{myo}$) and causes contraction. Relaxation is produced by reuptake of Ca^{2+} into the SR via the SR Ca^{2+} pump. Also shown is a channel in the longitudinal SR which is phosphate permeable and allows equilibration of the myoplasmic P_i ($[P_i]_{myo}$) with the SR P_i ($[P_i]_{SR}$). When the solubility product is exceeded calcium phosphate precipitates in the SR ($CaP_{i,ppt}$).

wave was found to be unchanged at the point of fatigue (8, 17, 104), indicating that there was little or no change in the ability of the AP to propagate along the surface membrane. In other cases (110), the M wave was found to be decreased in amplitude and area at the point of fatigue, although this does not necessarily mean that the AP would have been insufficient to trigger Ca^{2+} release and contraction within the fiber.

Many factors act in concert to reduce or counter the depolarizing effect of K^+ efflux in exercising muscle. Individual motor units are normally activated in vivo at a rate tailored to the fiber type and this is just sufficient for fused tetanic responses (14). Furthermore, the neural stimulation rate drops substantially during continuous or repeated muscle activation, yet still remains just adequate for full force production because the metabolic activity involved in the contractions changes the muscle properties in tandem, reducing the rate of relaxation and the fusion frequency (10, 17). These processes together minimize the number of APs involved in triggering contraction and hence also the associated K^+ efflux.

A further crucial aspect, which is frequently overlooked, is the major role played by Cl^- movements. Mammalian muscle fibers at rest are far more permeable to Cl^- than to K^+, with the chloride conductance being ~80% of the total (44, 70). This means that the resting membrane potential is strongly biased toward the Cl^- equilibrium potential (approximately −80 mV). Consequently, the depolarizing effect of acute increases in extracellular $[K^+]$ is greatly reduced. Although it is generally presumed that increases in extracellular $[K^+]$ will be particularly large and problematic within the T tubule, owing to its comparatively small volume, this is probably not the case. Most of the muscle Cl^- channels are located in the T tubule (34, 44), and part of the AP repolarizing current is likely carried by Cl^- influx and not just by K^+ efflux (48, 61). Furthermore, as the $[K^+]$ builds up in the T tubule, the electrochemical gradient for K^+ is reduced below the membrane potential and as a result K^+ is driven back into the cytoplasm through the inward rectifier K^+ channels (Kir2.1), which are present at high density in the T tubule (75, 117). In addition, the T-tubule membrane contains ~50% of the fiber's Na-K pumps, and these are strongly activated by both the rise in intracellular Na^+ and the muscle stimulation itself (32). These processes help counter K^+ accumulation in the T-tubule (48), with their task made easier by the comparatively small T-tubule volume.

Furthermore, the decreased intracellular pH that often occurs with muscle activity is not as deleterious to muscle performance as originally thought (for review, see Refs. 79, 124). In fact, it may help sustaining excitability by reducing the chloride conductance up to twofold, enabling APs to still propagate despite an appreciable level of fiber depolarization and Na^+ channel inactivation (91, 92).

The above factors together are possibly normally enough to prevent the loss of muscle excitability in exercising humans. Reductions in the size of the sarcolemmal AP are of little consequence provided that the AP is still sufficient to elicit an AP in the T tubule. Prolonged partial depolarization would be expected to reduce the size of the AP in the T tubule, but because the rate of repolarization of the AP is also slowed, the AP becomes prolonged and this tends to promote activation of the voltage-sensors opposing the effect of reduced AP amplitude. Depolarization also causes inactivation of a proportion of

the voltage sensors, rendering those sensors unable to open the Ca^{2+} release channels (87). Nevertheless, the overall efficacy of voltage-sensor coupling to Ca^{2+} release is also influenced by the Ca^{2+} release itself, which acts to *1*) shorten the AP, *2*) speed deactivation of the voltage sensors, and *3*) inactivate the release channels themselves (90). These negative feedback mechanisms on Ca^{2+} release, which together appear to help keep the amount of Ca^{2+} released by an AP relatively constant (96), even when the T tubule is substantially depolarized (47). As a result, peak tetanic force in fast-twitch muscle fibers is little affected unless the fibers are depolarized below approximately −60 mV (27), which is probably more extreme than would normally occur.

It is possible that after very prolonged exercise, Cl^- movements may eventually fail to adequately counter the effects of K^+ movements, owing to the continual influx of Cl^- and the consequent rundown of its electrochemical gradient. However, there is currently no evidence that this occurs. It will depend on whether the Na-K pump activity is sufficient in the long term to maintain an adequate $[K^+]$ gradient, and hence $[Cl^-]$ gradient, in the face of the ongoing, albeit reduced, rate of AP activity and K^+ efflux. Reduction of Na-K pump activity by reactive oxygen species may be a significant problem in some circumstances (86). Nevertheless, it appears that the fatigue observed in many human studies to date (see above), as well as in animal studies on isolated fibers (1), is probably not primarily due to AP failure or inadequate voltage-sensor activation, but instead is due to metabolic changes within the fibers that hinder Ca^{2+} release and contractile function.

MODIFICATION OF SR CALCIUM RELEASE

ATP, ADP, Mg^{2+}. In intense exercise in fast-twitch muscle fibers, ATP is consumed via the reaction ATP ↔ ADP + P_i at a much higher rate than it is regenerated aerobically. Initially, the cellular [ATP] is maintained at close to resting levels (~6 mM) and the [ADP] kept very low (~10 to 40 μM) by using the large supply of phosphocreatine (PCr; ~30–40 mM) to regenerate ATP via the creatine kinase (CK) reaction (PCr + ADP + H^+ ↔ Cr + ATP; Ref. 42, 118). The net reaction results in accumulation of both creatine (Cr) and P_i, as well as consumption of H^+. In addition, ATP is also resynthesized by anaerobic glycolysis, leading to the formation of lactate and H^+. If the ADP reaches a high enough local concentration, ATP is also regenerated by the myokinase reaction (2 ADP ↔ ATP + AMP), and most of the resulting AMP is rapidly deaminated to IMP (inosine monophosphate) by myoadenylate deaminase. The overall result is that the CrP is almost fully used early on, causing the [Cr] and $[P_i]$ to reach 30 mM or more, and the pH initially rises from its resting level (~7.0 to 7.1) and then declines substantially (often to ~6.6 or below) accompanied by an increase in [lactate], and at later times the [ATP] can decrease substantially with an almost equimolar increase in [IMP].

Early reports based on measurements in mixtures of fiber types concluded that [ATP] did not decline greatly during exercise, but recently Karatzaferi et al. (74) measured the metabolic changes that occurred in identified fiber types after maximal cycling exercise in humans over 25 s and showed that the [ATP] in the fastest fiber types (type IIX) dropped to ~20% (to a mean of ~5 μmol/g dry mass, or <1.8 mM ATP

expressed relative to cell water). This was the average level reached in the cytoplasm as a whole, and the level may have dropped even lower in local regions of high ATP consumption, such as near the myosin heads and the SR Ca^{2+} pumps or near the Na^+-K^+ pumps, which preferentially use local, glycolytically produced ATP. Because most ATP in a rested fiber has Mg^{2+} bound, and ADP, AMP, and IMP all have a much lower affinity for Mg^{2+}, the free $[Mg^{2+}]$ will rise as the [ATP] declines, increasing from ~1 to 2 mM or more (120).

If the ATP usage is great enough to substantially reduce the [ATP], voltage-sensor activation of the Ca^{2+} release channels is likely to be reduced. This occurs for several reasons. First, ATP needs to be bound to a cytoplasmic regulatory site on the release channels for them to be readily opened, with half maximal stimulation occurring at ~0.5 mM ATP (46, 81). Second, ADP and AMP act as weak competitive agonists and hence any accumulation of them near the release channels will antagonize the stimulatory action of the remaining ATP (46, 81). Finally, and perhaps of greatest importance, the Ca^{2+} release channel is also strongly inhibited by cytoplasmic Mg^{2+} (80) and voltage sensor-induced Ca^{2+} release is decreased ~40% if the $[Mg^{2+}]$ rises from 1 to 3 mM (18, 46, 120). Thus a combination of decreased [ATP] and increased $[Mg^{2+}]$ may inhibit voltage sensor-induced Ca^{2+} release during intense exercise in fast-twitch fibers (74). The inhibitory effects of increased $[Mg^{2+}]$ and reduced [ATP] on Ca^{2+} release also likely underlie the decrease in tetanic $[Ca^{2+}]_i$ observed in creatine kinase deficient ($CK^{-/-}$) mouse muscle fibers at the onset of high-intensity stimulation (37), although it is apparent that they are not the only metabolic factors involved in reducing Ca^{2+} release in fatigue (38).

A number of studies have reported that Ca^{2+} release from isolated SR is reduced up to ~40% following prolonged or intense exercise in humans (63, 83, 84). However, in such studies the Ca^{2+} release was triggered by non-physiological means, such as by caffeine, chloro-m-cresol, or the oxidizing agent Ag^+, and the rate of release was more than 30 times slower than occurs when activating Ca^{2+} release by the normal voltage sensor mechanism. Given that voltage sensor activation of Ca^{2+} release is frequently found to be unaffected by conditions that inhibit Ca^{2+} release in isolated SR channels (e.g., raised $[H^+]$, lactate, oxidation), it is unclear whether these observed exercise-induced changes in isolated SR vesicles reflect significant long-term inhibitory changes in Ca^{2+}-release channels.

In summary, the Ca^{2+} release channels play vital role during intense exercise by sensing any marked depletion of cellular ATP and responding by reducing Ca^{2+} release. This will decrease the rate of ATP usage because it reduces cross-bridge cycling and also SR Ca^{2+} uptake, the two main processes causing ATP hydrolysis. The result will be a reduction in power output, or in other words muscle fatigue, but the benefit is that it would help prevent complete exhaustion of all cellular ATP and consequent rigor development and cellular damage.

H^+ and lactate. Although low cytoplasmic pH inhibits Ca^{2+}- and caffeine-mediated activation of isolated Ca^{2+}-release channels, experiments on skinned fibers showed that the release channels in situ are readily opened by activating the voltage sensors, even at pH 6.2 (77, 78). Experiments on isolated mammalian fibers have also shown that low pH has little effect on tetanic force production at near physiological

temperature (125), nor does it noticeably affect the rate of fatigue development (23). Increasing the lactate concentration in the cytoplasm to 30 mM also has very little effect on twitch and tetanic force responses (95).

P_i. The equilibrium of the CK reaction is set so that during periods of fast energy consumption, the ATP concentration initially remains almost constant, whereas CrP breaks down to Cr and P_i. Cr has little effect on contractile function (89). P_i, on the other hand, decreases myofibrillar force production and Ca^{2+} sensitivity as well as SR Ca^{2+} release. Therefore, increased P_i is regarded as a major contributor to fatigue (124). We will now discuss mechanisms by which P_i can affect SR Ca^{2+} handling during fatigue.

An increase in tetanic $[Ca^{2+}]_{myo}$ is generally observed early during fatigue induced by repeated tetanic stimulation (Fig. 1*B*, *i* and *ii*). Experiments on $CK^{-/-}$ muscle fibers support a role of elevated P_i in this increase in tetanic $[Ca^{2+}]_{myo}$. When $CK^{-/-}$ muscle fibers were fatigued by repeated tetani, the early increase in tetanic $[Ca^{2+}]_{myo}$ did not occur (37, 38), but reappeared after CK injection into these fibers (35). Possible mechanisms by which elevated P_i can lead to increased tetanic $[Ca^{2+}]_{myo}$ include a direct stimulatory effect on the RyR (9) and/or inhibition or even reversing SR Ca^{2+} pumping (108, 111), which in the short term might increase tetanic $[Ca^{2+}]_{myo}$ (122). An important role of CK and P_i on SR Ca^{2+} pumping is supported by the finding that the early increase in tetanic $[Ca^{2+}]_{myo}$ during fatigue is accompanied by a marked increase in the amplitude of $[Ca^{2+}]_{myo}$ tails during relaxation in normal fibers, but this increase is not seen in $CK^{-/-}$ fibers (36).

Tetanic $[Ca^{2+}]_{myo}$ decreases as fatiguing stimulation progresses. CK and elevated P_i appear to play a central role in this decrease because it is markedly delayed in fibers with CK inhibition induced either pharmacologically (38) or genetically (37). Furthermore, CK injection into $CK^{-/-}$ fibers restored the normal decrease in tetanic $[Ca^{2+}]_{myo}$ in later stages of fatigue (35). Two mechanisms by which elevated P_i may decrease tetanic $[Ca^{2+}]_{myo}$ are a direct inhibition of the RyR and Ca^{2+}-P_i precipitation in the SR, causing a reduction of the amount of free Ca^{2+} available for release; this latter mechanism is discussed below in *Depletion of free calcium in the SR*. Skinned fiber experiments have shown an inhibitory effect of P_i on caffeine- and depolarization-induced SR Ca^{2+} release (43). Interestingly, this P_i-induced inhibition becomes more marked when Mg^{2+} is increased within the physiological range (43). Thus this Mg^{2+}-dependent P_i-induced inhibition of SR Ca^{2+} release fits with the inverse relationship between tetanic $[Ca^{2+}]_{myo}$ and $[Mg^{2+}]_{myo}$ during fatigue under normal conditions, which is completely lost after pharmacological inhibition of CK (38).

Reactive oxygen species. Production of reactive oxygen species (ROS) may increase substantially during intense muscle activity (for review see Ref. 66) and ROS have many cellular functions including protein modification by *S*-glutathionylation and disulphide bridge formation (for review see Ref. 60). ROS have long been suspected to have a role in muscle fatigue and their role has been established by experiments in which ROS scavengers reduce the rate of fatigue (88, 98, 107). However, clues to the mechanism of action of ROS in muscle fatigue are still limited.

A popular hypothesis is that ROS affect SR Ca^{2+} release (97), possibly by oxidative modification to one of the 100

cysteine residues on the RyR1. Early evidence for this possibility came from studies on isolated RyR1 in lipid bilayers in which the opening probability was increased by large (mM) concentrations of H_2O_2. Subsequently, many types of ROS have been shown to modify isolated RyR activity (for review see Ref. 52) and a recent study has identified 10 cysteine residues on RyR1 that undergo endogenous modification and a further three residues capable of modulation by exogenous ROS (6).

However, in intact muscle, RyR1 interacts with many other proteins and small molecules including the voltage sensor, calstabin1 (FKBP12), triadin, junctin, calsequestrin, calmodulin, ATP, ADP, Mg^{2+}, etc. Thus a critical question is whether, or how, the sensitivity to ROS persists in this more complex environment. Most of the available data suggest that in the intact environment SR Ca^{2+} release is relatively insensitive to the changes in ROS that occur under physiological conditions. For instance, in a study of rat skinned skeletal muscle with intact T tubules, Posterino et al. (94) found that H_2O_2 could increase SR Ca^{2+} release induced by caffeine or partial depolarization. But, critically, when SR Ca^{2+} release was induced by an AP, Ca^{2+} release appeared unaffected by either H_2O_2 or reducing agents such as DTT or reduced glutathione. The authors concluded that, under physiological conditions of Ca^{2+} release, the RyR channels rapidly reach essentially full opening and are consequently relatively insensitive to changes in the opening rate secondary to oxidative conditions. Studies in intact single fibers have led to the same conclusion. Andrade et al. (4, 5) applied H_2O_2 to single fibers and were able to observe effects on Ca^{2+} sensitivity of the myofibrillar proteins, but H_2O_2 had little effect on SR Ca^{2+} release and the observed increase in the tetanic $[Ca^{2+}]_{myo}$ appeared to be caused by slowing of the SR Ca^{2+} pump. Moopanar and Allen (88) observed no effect on tetanic $[Ca^{2+}]_{myo}$ in mouse muscle fatigued at 37°C, a condition in which the muscles fatigued rapidly by a ROS-dependent mechanism that was identified as reduced Ca^{2+}-sensitivity.

We conclude that, while RYR1 is clearly redox-sensitive, there is as yet no convincing evidence that ROS are modulating SR Ca^{2+} release during the induction of fatigue.

Glycogen. Glucose is stored as glycogen in skeletal muscle and this store is a major source of energy during most forms of muscle activity. A direct correlation between muscle glycogen concentration and time to fatigue during moderately intense exercise was first described by Hultman and coworkers (15) and has subsequently been confirmed in numerous studies. However, the mechanistic link between low glycogen and decreased force is not fully understood.

Experiments on mouse muscles with decreased glycogen stores have shown an accelerated decline of tetanic $[Ca^{2+}]_{myo}$ and hence force during fatiguing stimulation (30, 62). The link between reduced glycogen and decreased $[Ca^{2+}]_{myo}$ transients may relate to glycogen's central role in energy metabolism (62, 103) or it may be unrelated to energy metabolism. This latter possibility emerges from studies on toad muscle where the ability of skinned fibers to respond to T-tubular depolarizations correlated with the muscle glycogen content (nonsoluble component) even though ATP and PCr were present in the bathing solution (109). Also, the accelerated fatigue development in glycogen-depleted intact toad fibers was not associated with the normal decrease in the rapidly releasable SR Ca^{2+} store

(73). However, skinned rat EDL fibers showed only a small (11) or no (59) ATP- and PCr-independent effect of glycogen on the capacity to respond to depolarizations. Moreover, the faster fatigue development in glycogen depleted mouse muscle preparations was accompanied by normal changes in other fatigue-induced parameters (i.e., decreased myofibrillar Ca^{2+} sensitivity and maximum force, slowed relaxation, and increased resting $[Ca^{2+}]_{myo}$), which are generally attributed to metabolic changes (30, 62).

Phosphorylation reactions. Posttetanic potentiation, which may cause increased submaximal force production and faster contraction (12), is often observed after a brief period of muscle activity. The myosin regulatory light chain (RLC) is considered to have a central role in this potentiation because there is a correlation between the extent of myosin RLC phosphorylation and force increase (for review, see Ref. 112) and skinned fiber experiments have shown that myosin RLC phosphorylation increases force at submaximal, but not saturating, Ca^{2+} concentrations (93, 113). The myosin RLC phosphorylation is initiated by the increase in $[Ca^{2+}]_{myo}$ during contractions via a Ca^{2+}-calmodulin-dependent activation of skeletal muscle myosin light chain kinase (skMLCK; Refs. 112, 130).

Another Ca^{2+}-dependent kinase, calmodulin kinase II (CaMKII), may also be activated by the increase in $[Ca^{2+}]_{myo}$ during contractions. CaMKII is known to regulate several proteins involved in SR Ca^{2+} handling. In fast-twitch fibers, CaMKII may phosphorylate proteins involved in SR Ca^{2+} release, i.e., RyR, DHPR, and some of their associated proteins (33, 39). In slow-twitch fibers, CaMKII also acts on the SR Ca^{2+} ATPase and phospholamban (40, 102). CaMKII has been shown to be activated in exercising humans (100). Injection of CaMKII inhibitory peptide into fast-twitch mouse FDB fibers resulted in an ~20% decrease in tetanic $[Ca^{2+}]_{myo}$ in unfatigued fibers (114). Fatigue development was also accelerated by injection of the CaMKII inhibitory peptide or by application of pharmacological CaMKII inhibitor KN-93due to decreased SR Ca^{2+} release (7). Thus the fatigue-associated decline in $[Ca^{2+}]_{myo}$ and hence force would occur at a faster rate if they were not counteracted by Ca^{2+}-induced activation of skMLCK and CaMKII.

Exercise is generally associated with an increased activity in the sympathetic nervous system. β-adrenergic stimulation activates protein kinase A (PKA) in skeletal muscle resulting in phosphorylation of numerous skeletal muscle proteins (128), including RyR1 (99) and the Na^+-K^+ pumps (32). Acute adrenergic stimulation may increase skeletal muscle force production (19) and experiments with the β-adrenergic agonist terbutaline show that this may involve increased tetanic $[Ca^{2+}]_{myo}$, (28). Thus acute β-adrenergic stimulation of skeletal muscle cells may delay fatigue development by facilitating SR Ca^{2+} release. On the other hand, chronically increased β-adrenergic activity is associated with hyperphosphorylation of RyR1 and release of calstabin1 (also called FKBP12; Refs. 99, 119). Calstabin1 normally stabilizes RyR1 channels in the closed state and when calstabin1 is released, the channels become destabilized, resulting in increased SR Ca^{2+} leak and impaired muscle function (99). Thus while acute β-adrenergic stimulation may improve contractile function and increase fatigued resistance (19, 28, 128), prolonged stimulation may

have the opposite effect due to adverse effects on the RyR1 channel complex (99, 119).

DELAYED RECOVERY FROM FATIGUE

In 1977, Edwards et al. (50) described a very slow component of recovery from fatigue that can take days to recover. This type of fatigue is particularly prominent after contractions in which the muscle is stretched (eccentric contractions; Ref. 41). A characteristic of this type of fatigue is that recovery determined by tetani at high stimulus frequencies (50–100 Hz) is relatively fast ($t_{1/2}$ $\sim$5 min), whereas recovery at low frequencies (10–20 Hz) is much slower ($t_{1/2}$ 1–2 h). This type of fatigue occurs when metabolites have recovered and in the presence of normal APs (50). Studies in isolated mouse fibers have shown that delayed recovery can be caused by a reduction in tetanic $[Ca^{2+}]_{myo}$ at all frequencies of stimulation without changes in Ca^{2+} sensitivity (126). Elevation of tetanic $[Ca^{2+}]_{myo}$ with caffeine fully restored the force (31, 69, 126), indicating that the contractile apparatus was unaffected. The reduction in tetanic $[Ca^{2+}]_i$ was uniform throughout the single fiber, showing that it was not due to AP failure within the T tubule (126).

Several groups have demonstrated that if the resting $[Ca^{2+}]_{myo}$ is elevated for a prolonged period, a reduction in tetanic $[Ca^{2+}]_{myo}$ similar to that seen in delayed recovery can occur, suggesting that it is the elevated resting $[Ca^{2+}]_{myo}$ that triggers the impairment of Ca^{2+} release (24, 29). On the other hand, in a recent study fatigue was produced in the presence of N-benzyl-p-toluene sulphonamide (BTS), which inhibits cross-bridge force production (20), and the long-term depression of SR Ca^{2+} release was similar under these conditions despite the fact that in BTS considerable larger $[Ca^{2+}]_i$-time integral occurred during induction of fatigue. These and other results (31) suggest that both elevated $[Ca^{2+}]_{myo}$ and metabolic changes are required for development of delayed recovery. Another possibility is that there is some disruption of the T tubule/SR interaction caused by osmotic or mechanical stress associated with the repeated, and especially eccentric, contractions (22).

Experiments in skinned fibers with functional EC coupling show that coupling is disrupted if the cytoplasmic $[Ca^{2+}]$ is raised to tetanic levels (2–10 μM) for a prolonged period or to higher levels for shorter periods (76, 115). The effect is caused primarily by relatively high $[Ca^{2+}]$ near the release channels rather than by prolonged small rises in resting $[Ca^{2+}]$ (115). The reduced responses are not due to depolarization of the T tubule, nor to dysfunction of the release channels, which can still be fully activated by direct stimulation. Instead, the effect appears to be due to the voltage sensors failing to properly activate the release channels, quite possibly because the triad junctions become distorted following the Ca^{2+} exposure (76). The elevated $[Ca^{2+}]$ appears to induce its effect via a pH-dependent enzymatic reaction that does not involve phosphorylation or oxidation (76), but the exact mechanism is uncertain.

It has frequently been suggested that Ca^{2+}-dependent proteolysis may play a role in muscle fatigue and damage (13, 56). Skeletal muscle contains the ubiquitous calcium-dependent neutral proteases, μ-calpain and m-calpain, as well as a muscle specific isoform, calpain-3 (57). Ca^{2+}-dependent uncoupling is prevented in toad skinned fiber by leupeptin, a calpain inhibitor, but only at low $[Ca^{2+}]$ when the uncoupling proceeds

slowly (76, 116). Calpain inhibitors, however, were not found to prevent the prolonged failure of SR Ca^{2+} release in fatigued mouse fibers (29) or frog fibers (22). The variable effect of calpain inhibitors and the lack of obvious proteolysis of the DHPRs, RyRs, or triadin (76) coupled to the distortion of triad structures suggest a complex pattern of disruption involving target proteins within the triad that have yet to be identified.

In summary, the delayed recovery after fatigue is due primarily to a reduction in SR Ca^{2+} release. Prolonged, elevated resting $[Ca^{2+}]_{myo}$ may be involved in the induction of impaired coupling between the T-tubular voltage sensors and the SR Ca^{2+}-release channels, possibly involving calpains, but the exact mechanisms remain uncertain.

DEPLETION OF FREE CALCIUM IN THE SR

The decline of tetanic $[Ca^{2+}]_{myo}$ that has been observed in isolated fatigued muscles could arise from a reduction of the amount of Ca^{2+} stored in the SR. This possibility was first raised by Fryer et al. (54) on the basis of skinned fiber experiments in which a period of exposure to 50 mM myoplasmic phosphate caused a sustained reduction in SR Ca^{2+} release. They proposed that the P_i was able to enter the SR and that within the SR the $[Ca^{2+}][P_i]$ product exceeded the CaP_i solubility product (6 mM^2). Consequently Ca and P_i precipitated within the SR lowering the free Ca^{2+} ($[Ca^{2+}]_{SR}$) and reducing the amount of Ca^{2+} available for release (for review, see Ref. 3). This result has recently been extended to skinned fibers with intact EC coupling in which elevated P_i was shown to reduce the AP induced SR Ca^{2+} release (45).

A number of studies provide support for this proposal as a mechanism contributing to the reduced SR Ca^{2+} release in fatigue. First, injection of P_i into an unfatigued single fibers caused a striking fall in SR Ca^{2+} release probably because the P_i entered the SR and precipitated with Ca^{2+} (123). Second, in fatigued muscle fibers, the releasable Ca^{2+} in the SR, assessed by the rise of $[Ca^{2+}]_{myo}$ produced by rapid application of caffeine or 4-chloro-m-cresol, showed a decline during fatigue that recovered when the muscle was rested. If aerobic recovery was prevented during recovery by application of cyanide, an intervention which is known to prevent the recovery of P_i, it also prevented the recovery of the SR Ca^{2+} content (71). Third, $[Ca^{2+}]_{SR}$ can be measured by low affinity Ca^{2+} indicators introduced into the SR. Kabarra and Allen (72) used this technique to show that $[Ca^{2+}]_{SR}$ declined during fatigue and recovered during a subsequent rest period. Fourth, a P_i-permeable anion channel has been identified in the SR membrane (see Fig. 2), which could be the path for P_i entry and removal from the SR (82). Fifth, CK$^{-/-}$ muscles show a much smaller rise in P_i during fatigue (37). Such animals also show a slower rate of decline of tetanic $[Ca^{2+}]_{myo}$ during fatigue, consistent with the Ca-P_i precipitation hypothesis (Ref. 37; see also P_i).

While there is good support for this theory in muscle and in other tissues (55), there are a number of uncertainties. The time course with which P_i enters the SR is uncertain and the conditions within the SR microdomain may well influence the speed and extent of CaP_i precipitation. The main component of Ca^{2+} release recovers within minutes after fatigue induced by repeated tetani and, to confirm the CaP_i precipitation hypothesis, it will be important to establish both that precipitated CaP_i is present in the SR during fatigue and that it can resolublize

with a sufficiently rapid time course. A feature of muscle fatigue is that the rise in P_i occurs rapidly during fatigue (26), whereas the failure of SR Ca^{2+} release is typically a late feature of fatigue (Fig. 1). This could reflect slow entry of P_i into the SR or slow precipitation within the SR. Alternatively, because the P_i-permeable anion channel is inhibited by ATP (82), its open state may increase only slowly as ATP declines.

SR Ca^{2+} UPTAKE AND SLOWING OF RELAXATION

The ATP-driven SR Ca^{2+} pumps may be slowed by metabolic changes during fatigue. For instance, slowed pumping has been associated with decreased pH (51, 77, 106), increased P_i (108, 111), and decreased ATP/ADP·P_i ratio in the vicinity of the SR Ca^{2+} pumps (43, 108). In addition, the slowing may involve nonmetabolic factors because studies on SR preparations obtained from fatigued muscles show slowed Ca^{2+} pumping even when tested in standard solution (25, 58). This decline of SR Ca^{2+} pump rate is not, of itself, a cause of reduced SR Ca^{2+} release because slowing of the pump causes an immediate increase in tetanic $[Ca^{2+}]_{myo}$ (122). However, on a longer time scale, it might lead to reduced SR Ca^{2+} loading and release.

Slowing of relaxation is a hallmark of skeletal muscle fatigue. Fatigue in humans is often accompanied by a decrease in the motoneuron firing rate. Slowed relaxation may then limit the force decrease during isometric contractions by increasing the degree of mechanical fusion at lower stimulation frequencies (16, 67). On the other hand, slowed relaxation may limit the performance during dynamic exercise with alternating movements (1).

Relaxation of skeletal muscle cells involves the following major steps: 1) SR Ca^{2+} release stops; 2) Ca^{2+} is pumped back into the SR or bound to myoplasmic Ca^{2+} buffers, such as parvalbumin, provided these are not already saturated with Ca^{2+}; 3) Ca^{2+} dissociates from troponin; 4) cross-bridge cycling ends. Potentially, slowing of any of these steps can contribute to the slowing of relaxation. The knowledge about the relative importance of these steps to the fatigue-induced slowing of relaxation is limited because it is very difficult to study the individual steps in isolation and changes in them are likely to occur in parallel during induction of fatigue. Studies on fast-twitch mouse muscle showed slowed SR Ca^{2+} uptake in fatigue, but this was counteracted by decreased myofibrillar Ca^{2+} sensitivity and hence the reduced relaxation speed was ascribed to slowed cross-bridge cycling (121). Similar experiments on *Xenopus* frog muscle fibers, on the other hand, showed both a Ca^{2+}-related and a cross-bridge-related component in the fatigue-induced slowing of relaxation (127). A recent study on fatigue in human adductor pollicis muscles in vivo showed a strong temporal correlation between an increased curvature of the force-velocity relationship and slowed relaxation, which indicates that altered cross-bridge function contributed to the slowing of relaxation in this situation (68).

CONCLUSIONS

Impaired SR Ca^{2+} release is a well established mechanism in isolated muscles and contributes to several different types of fatigue. 1) Repeated short tetani (Fig. 1) have been investigated most intensively and it is clear that reduced SR Ca^{2+} release

contributes to the final phase (P3). We consider that the combined effects of reduced $[Ca^{2+}]_{SR}$ secondary to CaP_i precipitation and the inhibitory effect of high $[Mg^{2+}]_i$ and lowered [ATP] on the RyR1 are the most likely causes but there is no rigorous experimental evidence that can differentiate between these and other possibilities. 2) Continuous high frequency tetani also cause a decline in SR Ca^{2+} release and one component of this appears to be failure of the AP, probably in the T tubules. However, our interpretation of the literature is that this type of fatigue does not usually occur under physiological conditions because of a range of mechanism cooperate to minimize this phenomenon. Nevertheless disagreement is possible because there is no accepted method of determining the AP amplitude, particularly in the T tubules, in physiologically relevant fatigue models. 3) A delayed recovery of SR Ca^{2+} release can occur after intense and prolonged fatigue and gives rise to reduced force that is particularly apparent at low frequencies of stimulation. This type of impairment of Ca^{2+} release does not seem to be caused by either AP or metabolic changes and seems to be a consequence of some type of structural change in the voltage sensor or the ryanodine receptor or their interaction.

The techniques of rapid caffeine application and $[Ca^{2+}]_{myo}$ measurement have largely been restricted to isolated, and often single-fiber, muscle preparations. These preparations may differ from intact muscle in many respects, notably the high P_{O_2}, absence of extracellular K^+ accumulation, absence of many blood constituents (hormones, cytokines, plasma proteins, blood cells), absence of interaction with neighboring cells, artificially low temperature, etc. Since it is difficult to know which, if any, of these factors are important, it is essential to study fatigue in more complex and realistic models that still allow Ca^{2+} release to be assessed. In principle, rapid application of caffeine can be applied to blood-perfused preparations (64), although in these experiments only limited recovery of force was observed. This could mean that impaired SR Ca^{2+} release does not occur in intact blood-perfused preparations or it may be that fatigue had not reached phase 3, or the concentration of caffeine might have been insufficient. Several methods of measuring $[Ca^{2+}]_{myo}$ in blood-perfused muscles have been developed (65, 101) but have not been applied to fatigued muscles. We see the development of methods that can assess SR Ca^{2+} release in blood-perfused muscles and eventually in human and diseased muscles as important directions for the future.

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