invited review

Sarcoplasmic reticulum Ca^{2+} release and muscle fatigue

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Favero, Terence G. Sarcoplasmic reticulum Ca^{2+} release and muscle fatigue. *J. Appl. Physiol.* 87(2): 471–483, 1999.—Efforts to examine the relevant mechanisms involved in skeletal muscle fatigue are focusing on Ca^{2+} handling within the active muscle cell. It has been demonstrated time and again that reductions in sarcoplasmic reticulum (SR) Ca^{2+} release resulting from increased or intense muscle contraction will compromise tension development. This review seeks to accomplish two related goals: *1*) to provide an up-to-date molecular understanding of the Ca^{2+}-release process, with considerable attention devoted to the SR Ca^{2+} channel, including its associated proteins and their regulation by endogenous compounds; and *2*) to examine several putative mechanisms by which cellular alterations resulting from intense and/or prolonged contractile activity will modify SR Ca^{2+} release. The mechanisms that are likely candidates to explain the reductions in SR Ca^{2+} channel function following contractile activity include elevated Ca^{2+} concentrations, alterations in metabolic homeostasis within the "microcompartmentalized" triadic space, and modification by reactive oxygen species.

excitation-contraction coupling; muscle; calcium; exercise

PRODIGIOUS EFFORTS TO EXAMINE the relevant mechanisms involved in skeletal muscle fatigue have begun to focus on Ca^{2+} handling within the active muscle cell. It is understood that precise control of intracellular Ca^{2+} is central to optimal performance of skeletal muscle. First and foremost, activator Ca^{2+} released from the sarcoplasmic reticulum (SR) regulates cross-bridge interactions and thus controls force production. Reductions in SR Ca^{2+} release will primarily compromise tension development through reduced Ca^{2+} transients (49, 137, 140). Second, Ca^{2+}-activated tension and SR Ca^{2+} accumulation via the SR Ca^{2+}-ATPase consume a large fraction of the energy expenditure within the muscle cell (105). Thus regulation of intracellular Ca^{2+} fluxes can greatly influence muscle cell metabolism and may possibly serve as a link between the requirement for force and the metabolic state of the active muscle cell.

Eberstein and Sandow (43) first suggested inhibition of Ca^{2+} release from the SR as a likely and relevant factor in the fatigue process under certain experimental conditions. This has been confirmed time and time again by several different groups in a wide variety of species. Reductions in Ca^{2+} release have been noted in single muscle fibers from mouse (6), single whole (136) and cut fibers from frog (64), and whole bullfrog muscle (10). Moreover, inhibition of SR Ca^{2+} release was noted in the rat after it run to exhaustion (46). (For a more detailed account of the muscle preparations and fatigue protocols please see Refs. 49, 131, 133, and 137.)

Inadequate or suboptimal delivery of Ca^{2+} to the myofilaments via SR Ca^{2+} release could occur under three scenarios: *1*) impaired coupling between the voltage sensors in the t-tubule (TT) membrane and the SR Ca^{2+} channel [excitation-contraction coupling (ECC)], *2*) reductions in or modification of the SR Ca^{2+} content, or *3*) by transient modification of the SR Ca^{2+} channel, reducing its opening probability after activation (64, 137).

It is difficult to assess the contributions of the first hypothesis, considering that the complete mechanism for ECC has yet to be adequately defined. However, Gyorke (64) has suggested that fatigue from tetanic stimulation does not modify function of the t-tubule voltage sensor and is localized to the SR Ca^{2+} channel.

It is possible that SR Ca^{2+} content becomes diminished, considering that the catalytic activity of the SR Ca^{2+}-ATPase is inhibited by a single bout of muscle activity (22, 78, 135) or that "releasable" Ca^{2+} may be buffered by luminal phosphate that becomes elevated in skeletal muscle undergoing activity (55, 103, 132). This area will be addressed in BIOPHYSICAL REGULATION OF SR Ca^{2+} RELEASE.

Clearly, the most studied and well-supported contention suggests that activity-induced reductions in myoplasmic Ca^{2+} transients are mediated by the SR Ca^{2+} channel and/or its related regulatory proteins. An early observation by Eberstein and Sandow (43) showed that the application of caffeine, a Ca^{2+}-channel agonist, reversed the loss of force production in fatigued fibers.

This result, confirmed many times (58, 62, 87, 98), pointedly implicates modification of the SR Ca^{2+} channel during fatiguing protocols.

Whereas SR Ca^{2+} channel dysfunction may not be the only cause of fatigue under all conditions of muscle activity, it is considered a viable mechanism for a several different muscle activation patterns. A myriad of studies have demonstrated alterations in myoplasmic Ca^{2+} during and/or after muscle activity. More complete reviews detailing the various fatigue protocols as well as contributions from the myofilaments and the influence of metabolism have been expertly reviewed elsewhere (49, 120, 137, 140). In light of these works, this review seeks to accomplish two related goals: *1*) to provide an up-to-date molecular understanding of the Ca^{2+} release process, with considerable attention devoted to the SR Ca^{2+} release channel, its associated proteins, and channel regulation by endogenous compounds; and *2*) to examine several putative mechanisms by which cellular alterations resulting from intense and/or prolonged contractile activity will modify SR Ca^{2+} release. This review is not intended to be a complete account of the many studies produced over the past decade. Thus apologies are extended for the exclusion of any work that supports this current undertaking.

ECC AND THE SR CHANNEL

ECC is a sequential process that begins with activation of the muscle cell membrane and concludes with Ca^{2+}-activated tension produced by the myofilaments. Several key proteins participate in this process: the dihydropyridine (DHP) receptor, triadin, a 12-kDa FK506-binding protein (FKBP12), calsequestrin, calmodulin, junctin, and the SR Ca^{2+}-release channel-ryanodine receptor (RYR).

DHP receptor. The skeletal muscle DHP receptor is an oligomeric complex composed of five different subunits ($\alpha1$, $\alpha2$, β, δ, and γ) (27). All five subunits have cytoplasmic domains, yet the $\alpha1$-subunit appears to be the most important. It contains the binding site for DHPs and other antagonists and also can form an L-type Ca^{2+} channel (26), but Ca^{2+} entry is not required for activation of the SR Ca^{2+}-release channel. It is currently believed that the DHP receptors found within the t tubule serve as the voltage sensors associated with asymmetric charge movement, an event consistent with depolarization to the contractile threshold (109). The $\alpha1$-subunit consists of four repeats with three cytoplasmic loops between each segment (27). The II-III loop has been postulated to have a role in communication between the DHP and RYR receptors, as peptide fragments from the II-III loop of the DHP receptor bound to and activated the purified skeletal RYR Ca^{2+} channel but had no effect on the cardiac receptor (90). Both the $\alpha1$- and β-subunits contain multiple sites for phosphorylation that may be critical during muscle activity. The functions of the other subunits have not been fully defined.

Triadin. Several proteins have been localized in the triadic region and are thought to participate in the interaction between the DHP receptor and the SR Ca^{2+}-release channel; among them, triadin, a 95-kDa glycoprotein, has been the most thoroughly studied (19, 20). The most compelling evidence arguing for its participation in ECC is that triadin binds to both the RYR channel and the DHP receptor in protein overlay studies, although no reports of direct in vivo interactions have been published (79). In other studies, triadin has been shown to directly interact with the RYR Ca^{2+} channel via hyperreactive thiols when the channel is in the open configuration (92). Cloning of a protein that appears to be identical to triadin (80) indicates that the protein contains one transmembrane domain and a large luminal component. The luminal region contains a number of positively charged residues and may serve as an anchoring protein for calsequestrin, the moderate affinity-high capacity Ca^{2+}-binding protein that has been implicated in modifying SR Ca^{2+} release (69). Continued work should elucidate a more concrete role for triadin.

FKBP12. Four low-molecular-weight binding proteins are associated with each functional RYR channel. These FKBPs function as receptors for the immunosuppressant drugs, FK506 and rapamycin. When these drugs are added to muscle fiber or SR preparations, they induce dissociation of the FKBP12 proteins and appear to reduce or relieve channel inhibition by Mg^{2+} (4), H^+ (127), and possibly millimolar Ca^{2+} (42). In single-fiber experiments, removal of FKPB12s by rapamycin potentiated Ca^{2+} release evoked by depolarization or caffeine application (86). Interestingly, repeated depolarizations resulted in an irreversible loss of depolarization-induced release but not caffeine-induced activation of the release channel. Although the removal of these proteins appears to affect native channel function, it is not known how the proteins participate in normal channel gating or are modified by extended contractile activity.

Calmodulin. Calmodulin is a cytoplasmic Ca^{2+}-dependent protein that shares many similarities with troponin (28). Upon binding up to four Ca^{2+}, the Ca^{2+}-calmodulin complex can control a large number of calmodulin-dependent enzymes and membrane transport proteins. By binding to the skeletal muscle RYR protein, calmodulin has been shown to modulate channel activity (129). At low Ca^{2+} concentration (submicromolar), calmodulin stimulates Ca^{2+} flux and increases single-channel gating activity (129). At micro- to millimolar concentrations, calmodulin inhibited the RYR severalfold, thus suggesting that calmodulin may play a role in both activation and inhibition of SR Ca^{2+} release (129).

Calsequestrin. Calsequestrin is a low-affinity, high-capacity Ca^{2+}-binding protein that resides within the SR lumen. It has been shown to be anchored to the junctional SR membrane, thus ensuring a large quantity of Ca^{2+} near the release channel (35). It does not bind to the RYR, but has been shown to bind to triadin (63) and has been implicated in modifying Ca^{2+} release from the SR (39, 69).

Junctin. Junctin was first discovered in canine heart muscle and later in skeletal muscle (76). Amino acid

sequencing reveals that, like triadin, it resides on the luminal side of the SR membrane (76). It has recently been shown to bind to triadin, calsequestrin, and the RYR protein (145) and is thus believed to form a luminal complex that regulates SR. It appears that junctin and triadin interact directly in the junctional SR membrane and stabilize a complex that anchors calsequestrin to the RYR. Taken together, these results suggest that junctin, calsequestrin, triadin, and the RYR form a quaternary complex that may be required for normal operation of Ca^{2+} release.

SR RYR Ca^{2+}-release channel junctional foot protein. This protein is the critical component in ECC, as Ca^{2+} release is lost in transgenic mice lacking the RYR protein (123). Originally isolated as the junctional foot protein (77), it was later determined to be the SR Ca^{2+}-release channel from morphological and pharmacological studies with ryanodine (70, 94, 102). Because the protein has been identified by different methods, it has acquired several names that are frequently used interchangeably. The skeletal muscle RYR is a high-molecular-weight homotetramer containing four subunits. The monomeric protein contains 5,035 amino acids, with an apparent molecular mass of 560 kDa (124). The native protein that has been visualized by electron microscopy appears as a quatrefoil structure (cloverleaf-like) (104). Sequence analysis suggests the presence of cytoplasmic Ca^{2+}, calmodulin, and adenine nucleotide binding as well as phosphorylation sites.

Three mammalian isoforms have been identified and are encoded by three different genes, *ryr*1, *ryr*2, and *ryr*3. The RYR1, RYR2, and RYR3 proteins correspond to the skeletal, cardiac, and brain isoforms, respectively, the order in which they were identified. Brain tissue contains small amounts of the skeletal and cardiac forms as well as its native protein, whereas the muscle forms predominate in their respective tissues (65). A more extensive and detailed discussion of this protein has been published by Coronado et al. (34).

Physical arrangement of proteins. Morphological analysis using immunolabeling and electron microscopic studies revealed that the DHP receptor and RYR-Ca^{2+} channel were localized in the triadic (two terminal cisternae abutted up to one t tubule) region of mammalian skeletal muscle. The most simplistic arrangement for purposes of ECC would suggest that every DHP receptor reside directly opposite a RYR-Ca^{2+} channel. This, however, does not appear to be the case. Four particle clusters, or tetrads, thought to represent four DHP receptors, were located opposite and within close proximity (10–15 nM) to every second junctional RYR, creating a checkerboard pattern and revealing a possible direct link between at least one-half of the RYRs (16). Recent work also suggests that as many as 20% of RYRs have been located outside the specific triad area, but their function has not been determined (40). The aforementioned proteins such as triadin, calsequestrin, FKBP12, and junctin are believed to be localized near the RYR foot structure, but the exact placement and consequent function of these proteins remain obscure. In addition, two glycolytic enzymes, glyceraldehyde 3-phosphate dehydrogenase and aldolase, co-sediment with triad-enriched SR vesicles (25, 66).

Despite the advances in mapping the triadic region of skeletal muscle, clarity has not been achieved (52). Moreover, our current understanding of the ultrastructural map does not provide much information as to the accessibility of the triadic space by diffusable mediators that may modify function. For example, single-channel analysis suggests that alterations in the H^+ concentrations can significantly alter channel gating (110). However, this result is not confirmed in single-fiber studies with intact t tubules, where pH manipulations had no effect on SR Ca^{2+} release (31, 84). Accurate determination of the biophysical architecture leading to a better understanding of its pharmacological and biochemical constraints should provide a more complete list of those metabolites that participate in the SR Ca^{2+}-release process during muscle activity.

CURRENT THEORIES IN ECC

Many cell types are activated by second-messenger systems following receptor-mediated signaling. Smooth muscle and neurons utilize inositol 1,4,5-triphosphate (IP_3) to promote the release of Ca^{2+}. Whereas several early studies suggested that IP_3 may modulate SR Ca^{2+} release in skeletal muscle, its inability to rapidly activate skinned fibers argues against IP_3 as the main signaling protein in skeletal muscle (see Ref. 93 for discussion).

The ECC processes are different between mammalian skeletal and cardiac muscle. In heart tissue, the DHP receptor, an L-type Ca^{2+} channel embedded in the t-tubule membrane, mediates the influx of Ca^{2+} following an action potential and voltage-dependent activation. The influx of extracellular Ca^{2+} into the region of the closely opposed SR Ca^{2+} channels then induces a large increase in intracellular Ca^{2+} mediated by the SR channel (138). This process is well defined and has been aptly named calcium-induced calcium release (CICR). In skeletal muscle, Ca^{2+} entry is not required to activate the SR Ca^{2+}-release process, as chelation of t-tubule Ca^{2+} does not prevent activation of the RYR channel and SR Ca^{2+} release (8, 41).

In skeletal muscle, a mechanical coupling was proposed by Schneider and Chandler (113), as they suggested that charged molecules located in the t-tubule membrane move during depolarization, and these molecules provide a physical link between the t tubule and the SR Ca^{2+}-release process. They observed that voltage-dependent charge movement was closely associated with muscle contraction and could be measured across the t-tubule membrane when all ionic currents were blocked. Subsequent experiments showed that DHPs blocked charge movements and SR Ca^{2+} release with similar voltage and dose dependencies, thus arguing for a critical role of the DHP receptor in the activation of Ca^{2+} release (109). This sequence was later confirmed, as dysgenic myotubes lacking the DHP receptor α1-subunit failed to elicit requisite charge movement

and ECC (11). This coupling was restored following transfection of the α1-unit (123, 125).

The current hypothetical mechanism suggests a direct interaction between the DHP-receptor tetrads and its closely associated RYR proteins, where voltage-dependent activation of the DHP-receptor proteins triggers Ca^{2+} efflux via the RYR (109). As Ca^{2+} exits the mouth of the channel, it putatively diffuses laterally to the unlinked RYR channels providing a role for Ca^{2+} ion activation or potentiation of the release process (109). Myoplasmic Ca^{2+} rises rapidly from ~50 nmol/l to 1–5 μmol/l, a concentration great enough to saturate the troponin-binding sites allowing interaction between actin and myosin.

The RYR displays a biphasic Ca^{2+}-dependent behavior as submicromolar Ca^{2+} potentiates Ca^{2+} channel opening while millimolar Ca^{2+} induces closing of the RYR channel. Ca^{2+} ions in ECC and SR release have received much interest (96). Ca^{2+} has long been proposed as an activator in the Ca^{2+}-release process, as the increases in myoplasmic Ca^{2+} after depolarization are thought to produce a positive-feedback CICR. This may be the critical trigger for those channels not directly coupled to t-tubule voltage sensors (109, 143). Inhibition of RYR activity at high concentrations of Ca^{2+} has been suggested to close the RYR after activation (109). However, this hypothetical mechanism does not explain how the SR channel maintains its closed configuration as the myoplasmic or triadic Ca^{2+} concentrations are lowered back to nanomolar level during SR Ca^{2+}-ATPase sequestration following cessation of excitation. Although Ca^{2+} ions may not be the direct trigger of SR Ca^{2+} release, it is difficult to rule out the possible contributions they may have to potentiating SR Ca^{2+} release in vivo or under conditions of prolonged contractile activity.

Recently, Stephenson et al. (120) have argued an important role for Mg^{2+} in regulation of SR Ca^{2+} release. Their model suggests that by binding to the low-affinity Ca^{2+}-binding site, Mg^{2+} (1 mM) exerts a strong inhibitory effect on channel activity, essentially closing the channel during resting conditions (85). They propose that "coupling" between the DHP receptor and RYR induces dissociation of Mg^{2+}, via FKBP12 participation, removing the inhibition allowing Ca^{2+} to exit the channel and thus more fully activating the RYR and its neighboring unlinked RYRs (85). Under their assertion, Ca^{2+} release is controlled by the voltage sensors on the DHP receptor that regulate Mg^{2+} binding and dissociation from the RYR channel.

It has also been proposed that endogenous sulfhydryl (SH) groups present on the RYR protein may be responsible for regulating the gating of the SR Ca^{2+}-release channel (1, 111, 112). It has been demonstrated that oxidation of the SH groups stimulates SR Ca^{2+} release in vesicle experiments (2) and muscle fibers (112). Whereas no clear role for the SH regulation has been established, it remains a viable mechanism to modulate channel gating.

Despite considerable effort over the past decade, the mechanism that describes SR Ca^{2+} channel activation is unknown. Although the DHP receptor is voltage regulated, the SR Ca^{2+} channel does not display voltage sensitivity, nor does it appear to be electrically coupled to the DHP receptor. Several hypotheses have suggested that various proteins participate in this process. Roles for triadin and the II-III cytoplasmic loop from the DHP receptor α1-subunit have been proposed but not completely elucidated.

In summary, the following sequence of events is currently thought to explain ECC: *1*) sarcolemmal action potential, *2*) TT charge movement, *3*) coupling of TT charge movement to the SR Ca^{2+}-release channel, *4*) Ca^{2+} release from the SR, *5*) Ca^{2+} binding to troponin, and *6*) actomyosin ATP hydrolysis and cross-bridge cycling. As discussed above, because sequestration of Ca^{2+} ultimately restores the Ca^{2+} gradient, thus "loading" the SR membrane, the uptake of Ca^{2+} may be included as an accessory part of the ECC mechanism.

SR CA^{2+} RELEASE FROM AN EXPERIMENTAL PERSPECTIVE

When evaluating SR Ca^{2+} channel function, it is important to understand and evaluate the techniques from which the data were derived. Much of our current understanding regarding channel function was elucidated through the application of three different techniques (discussed below). Concurrent with the development and application of techniques used to study channel function was the identification of a variety of chemical modulators that can regulate the RYR Ca^{2+} channel activity. The list of activators of SR Ca^{2+} release includes anthraquinones, halothane, fatty acid derivatives, ryanodine, Ca^{2+}, adenine nucleotides, caffeine, oxidizing reagents such as hydrogen peroxide, and sulfhydryl reagents (see Table 1 for specific concentrations). Paralleling the list of activators was the identification of compounds that inhibit Ca^{2+} release. Among them are the polycationic dye ruthenium red; lactate, both Ca^{2+} and Mg^{2+} in the millimolar range; and reducing agents, such as dithiothreitol and glutathione. Whereas many of these compounds are not physiological, several have proved extremely useful in describing channel regulation following physiological modification. In particular, experiments with caffeine and ryanodine have led to descriptions of channel

Table 1. *Modulators of the SR Ca^{2+} release channel*

Stimulators	Concentration	Inhibitors	Concentration
Ca^{2+}	0.1–100 μM	Ca^{2+}	>1 mM
Adenine nucleotides	1–5 mM	Mg^{2+}	0.5–5 mM
Caffeine	>0.5 mM	Ruthenium red	0.01–20 μM
Ryanodine	5–40 mM	Ryanodine	>10 μM
Ag^{+}	5–50 μM	Calmodulin	0.2 μM
Cu^{+}/cysteine	2/10 μM	Lactate	10–20 mM
Reactive disulfides	1–20 μM	Procaine	1–20 mM
Doxorubicin	5–50 μM	Tetracaine	0.1–2 mM
Peroxide	1–100 mM	CPM	10–100 nM
Porphyrins	1–60 μM	Neomycin	0.01–20 μM
GSSG	0.52–2 mM	Reducing agents	1–5 mM

Modified from Coronado et al. (34). CPM, 7-diethylamino-3(4′-maleimidylphenyl)-4-methylcoumarin.

behavior that is much like a "fingerprint," indicative of a certain type of channel dysfunction. As these reagents become more widely utilized, our ability to detect important, and yet subtle, changes in channel function will be advanced.

Vesicle flux assays. The majority of the early studies examined SR Ca^{2+} release by using isolated SR vesicle preparations (2, 94, 111). These vesicles could be actively loaded in the presence of Ca^{2+} and ATP, developing a 3,000-fold Ca^{2+} gradient across the SR vesicle. Once loaded, a wide variety of reagents can be used to trigger Ca^{2+} efflux. SR vesicle flux experiments are advantageous because they closely parallel in vivo Ca^{2+} release, as a large gradient provides the driving force for release. Triad preparations can also demonstrate activation by t-tubule depolarization following ionic substitution (7). The greatest advantage of this assay is that it yields representative data by averaging the kinetic behavior of a considerable number of channels contained by the vesicles. However, several drawbacks detract from the global utility of this assay. SR isolation is time consuming, it is possible that key SR proteins are lost as a result of the lengthy procedure, and, last, these assays do require significant amounts of protein, usually requiring larger muscle samples (~1 g muscle needed to isolate SR, the yield is ~1 mg/g muscle). Furthermore, because the assays are performed under "active" loading conditions where extracellular Ca^{2+} is monitored, it is possible that reagents that affect Ca^{2+}-ATPase activity or modify membrane permeability can influence Ca^{2+} movements independent of SR Ca^{2+} release. For example, it is very difficult to perform SR Ca^{2+} release assays at different H^+ concentrations, as Ca^{2+} loading is significantly inhibited at lower pH values, thus reducing the driving force for SR release.

Ryanodine-binding assays. Our greatest understanding of release channel regulation was advanced by the development of the ligand-binding assay using tritiated ryanodine (50, 94, 102). Ryanodine is a neutral plant alkaloid and a naturally occurring insecticide (72) that binds with nanomolar affinity to open Ca^{2+} channels. The greatest benefit of this assay is that ryanodine binds specifically to its receptor (the RYR-release channel) and to no other protein in the SR. With few exceptions, compounds that activate the SR Ca^{2+} channel and stimulate Ca^{2+} release also enhance the binding of ryanodine. The opposite is true for Mg^{2+}, as it reduces SR Ca^{2+} release in a concentration-dependent manner and inhibits the binding of the ryanodine to its receptor. These macroscopic assays are not technically difficult, and they provide ample opportunity to manipulate a wide variety of conditions that would be difficult to carry out in flux assays. However, the binding is quite slow (typically 3 h) and thus it does not provide temporal data consistent with SR Ca^{2+} release in single-fiber or vesicle experiments.

Single-channel analysis. By fusing isolated SR vesicles to lipid bilayer membranes, the SR Ca^{2+} gating characteristics can be evaluated (117). Much of the pharmacological and biochemical data generated from the two macroscopic assays have been confirmed on the single-channel level by using isolated proteins. The fused protein remains sensitive to almost all of the known activators and inhibitors of Ca^{2+} release and ryanodine binding. The channel is permeable to both mono- and divalent cations, but because the channel is almost three times more permeable to Cs^+ than Ca^{2+}, Cs^+ is frequently used as the charge carrier. Following fusion and incorporation of the Ca^{2+}-release channel into the bilayer membrane, both the cytoplasmic and luminal sides of the channel protein can be manipulated. This technique is technically demanding, but direct access to a single-gating channel can provide important and detailed information on a molecular level. However, this technique may be self-limiting where macroscopic assays might provide more conclusive data. For example, it was previously determined that SR Ca^{2+} release was reduced by 30% after a run to exhaustion in rats (46). It is believed that as few as one RYR channel exists per vesicle (some vesicles contain no channels), and if only 30% of the channels are dysfunctional, it is possible that 70% of the fusion events will contain unmodified RYR proteins. Thus many assays would have to be conducted to ensure that the stated effect was observable at the single-channel level. Still, this technique was used to determine the Ca^{2+} sensitivity of gating RYR channels isolated from denervated rat muscle (38). In the native state, Ca^{2+} channel experiments are performed as many as six to ten times to confirm channel behavior.

The physiological relevance of the above biochemical and pharmacological characterizations have been advanced and clarified in single-fiber studies. From these studies, it appears as if the Ca^{2+}-release process may be dually regulated (85). DHP receptor-mediated activation exerts the most important control over the SR Ca^{2+}-release process. However, modulation of SR Ca^{2+} release can be accomplished through a variety of reagents that are known to stimulate or inhibit RYR activity. Considering this evidence, it is important to evaluate reagents typically used in both biochemical and various fiber preparations. Some, but not all, ligands that modulate RYR channel activity in SR vesicle experiments perform similarly at the level of a single muscle fiber. Lamb and Stephenson (85), using skinned fibers with intact TT, have shown that after a normal depolarization Ca^{2+} release and muscle contraction could be completely inhibited by high Mg^{2+} concentrations, a result consistent with SR vesicle studies (95). They demonstrate that the function of the SR Ca^{2+} channel is controlled by the TT under resting conditions but it is also sensitive to the changing intracellular milieu. On the other hand, in a different study, Lamb et al. (84) demonstrated that reductions in intracellular pH have little effect on ECC in preparations where Ca^{2+} release is controlled by native voltage activation mediated by the DHP receptor. These two studies should serve as a caveat when attempting to support results from single-fiber experiments with data obtained from SR vesicle or single-channel preparations.

BIOPHYSICAL REGULATION OF SR Ca^{2+} RELEASE

In addition to the chemical/mechanical/electrical mechanisms that govern ECC, release of Ca^{2+} from the SR lumen is dependent on the physical constraints of the ion channel and luminal ion concentration. A reduction in Ca^{2+} ion flux through the channel may be affected by altering the gating characteristics, modification of the channel conductance, and/or by a reduction in the SR lumen/cytoplasm Ca^{2+} gradient that determines the driving force for release.

The SR channel gates in a two-state (open or closed) model. The two primary gating characteristics that determine ion flow are the probability that the channel is in the open state (P_o) and mean open time, the average time the channel remains in the open configuration once open. On the single-channel level, compounds that close the channel generally do so by reducing the P_o. This may be accomplished by reducing the time spent in the open configuration or reducing the frequency of opening. This behavior has been observed by several laboratories, with many of the compounds listed in Table 1. For example, Smith et al. (117) demonstrated this by using Mg^{2+} to inhibit the SR release channel.

Whereas it is theoretically possible that the channel conductance may be modified, this scenario is unlikely during fatigue. There are very few examples in which conductance has been modified by anything other than nonbiological organic molecules. Ryanodine can induce subconductance states in single-channel experiments (68, 82). Furthermore, subconductance behavior was also recently reported after the removal of FKBP12 by rapamycin (4), but this was also accompanied by an increase in the P_o, making it difficult to extrapolate these results to conditions of muscle fatigue.

Recent work indicates that the SR lumen/cytoplasmic Ca^{2+} gradient may become diminished as a result of either inadequate refilling following Ca^{2+} release or by reducing the amount of free releasable Ca^{2+} stored within the lumen.

The total SR Ca^{2+} content has been determined to be 1–2 mM per fiber volume (51, 55) and represents the driving force for Ca^{2+} release into the myoplasm. Fryer et al. (56) have indicated that SR Ca^{2+} content is different between fast and slow type muscle. Luminal Ca^{2+} content is dependent on several factors including, but not limited to, function of the SR Ca^{2+} ATPase, leakiness of the SR membrane and/or RYR channel, and the total amount of Ca^{2+} released that is a function of duration and frequency of the contractile protocol. Inhibition of the SR ATPase (22, 91) and reductions in Ca^{2+} accumulation by SR have been demonstrated after exhaustive running in rats (22, 78), in single-fiber experiments (135, 141), and in exercising humans (17). Evidence suggests that with prolonged stimulation resting intracellular Ca^{2+} concentrations are significantly elevated (89). However, one early report suggested that SR Ca^{2+} content is not modified (61).

Reductions in the amount of releasable SR Ca^{2+} due to the formation of a Ca^{2+}-P_i precipitate would also decrease SR Ca^{2+} release, as has been suggested by Fryer et al. (55). P_i can rise to 30–40 mM in the active muscle cell (23), a concentration sufficiently high enough for P_i to enter the SR and complex with Ca^{2+} forming a precipitate (57). Recent work from Fryer et al. (55, 57) and Westerblad and Allen (132) indicates that physiological concentrations of P_i will reduce force by decreasing SR Ca^{2+} release, an event whose time course of recovery is consistent with the resolubilization of the Ca^{2+}-P_i precipitate (103). Continued work in this area should reveal more evidence detailing the implications of reduced SR Ca^{2+} content and/or the effects of P_i on SR Ca^{2+} concentrations during muscle fatigue. Further study should help to clarify the effect of prolonged contractile activity on SR Ca^{2+} load and loading parameters.

FAILURE OF SR Ca^{2+} RELEASE AND MUSCLE FATIGUE

Intense or prolonged contractile activity can reduce muscle force output that recovers after the cessation of activity. This sequential pattern of events is typically referred to as muscle fatigue. In part, the length of the recovery period is one of several characteristics that distinguish different types of fatigue, in particular, low-frequency fatigue (LFF) and high-frequency fatigue (HFF) (74, 75). HFF is characterized by a rapid loss of force that recovers very quickly once muscle activation is reduced or stopped (75). It has been suggested that HFF is most likely due to disturbances in muscle excitation related to ionic imbalances that are readily reversible (24, 75). It has been questioned whether this type of fatigue is normal in healthy muscle (75). Conversely, LFF, first described by Edwards et al. (44), is characterized by a loss of force primarily at lower stimulation frequencies, from which recovery is slow and may persist for hours or days, despite the absence of any gross disturbances in the muscle (75). It is important to point out that this type of fatigue can be caused by a variety of forms of activity, but loss of force is noticeable at lower stimulation frequencies. It has been suggested that LFF may be due to a wider variety of mechanisms including, but not limited to, metabolic, mitochondrial, and/or SR disruptions. Despite the fact that the etiology of muscle fatigue is very dependent on that stimulation frequency or muscle activation protocol, along with other considerations such as fiber type, state of training, etc., SR Ca^{2+} release remains a focal point.

The type of stimulation protocol that is most like prolonged submaximal exercise is LFF. Fatigue induced by low-frequency stimulation tends to follow a stereotypical pattern of force decline (133). During the initial phase, force declines rapidly to ~80% of control. During the second phase, a more stable force production pattern predominates, with a slight additional reduction in force to 70%. This is followed by a rapid decline of force to ~30–40% and is referred to as "phase three." It has long been suggested that reductions in SR Ca^{2+} release were involved in loss of force observed at low stimulation frequencies (44) and are suggested to be the primary factor in phase-three fatigue (133).

Currently, several mechanisms are likely candidates to explain the reductions in SR Ca^{2+} channel activity following contractile activity. They include, but are not limited to, elevated Ca^{2+} concentrations, alterations in metabolic homeostasis within the "microcompartmentalized" triadic space, and modification by reactive oxygen species (ROS).

Elevated Ca^{2+}. Westerblad et al. (135) have suggested that reductions in force, particularly LFF, can be explained by a reduction in the SR Ca^{2+} release. Consistent with a reduction in Ca^{2+} release is a general loss of Ca^{2+} homeostasis where intracellular Ca^{2+} is elevated significantly. Lamb et al. (83) have shown that raised intracellular Ca^{2+} in skinned fibers abolishes ECC and alters the structure of the t-tubule SR triad. Reductions in Ca^{2+} release after exposure to 100 μM Ca^{2+} were noted to be time dependent (longer exposures induce greater uncoupling), temperature dependent (uncoupling 13-fold slower at 3°C compared with 23°C), and pH dependent (slower at lower pH values). Interestingly, Mg^{2+} elevated to 10 mM did not prevent or induce uncoupling at high Ca^{2+}. Whereas these studies focus on ECC as a whole, they do not pinpoint the critical step that is modified by elevated Ca^{2+}. This, however, was examined by Williams (139), who recently demonstrated that in skinned frog fibers SR Ca^{2+} release is directly affected by elevated Ca^{2+}. A single 5-min exposure to 500 nM Ca^{2+}, a concentration noted to accompany fatiguing stimulation (135), reduced caffeine-stimulated SR Ca^{2+} release. Chin and Allen (29) and Chin et al. (32) have further characterized this phenomenon. Their work suggests that the duration of exposure must be considered along with the level to which intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is raised, arguing that the "Ca^{2+}-time integral" is most critical in predicting the deficit in SR Ca^{2+} release induced by raise in intracellular Ca^{2+} (32).

It has been suggested that under these conditions Ca^{2+} release may be modified by proteolytic digestion of key SR proteins. Skeletal muscle contains significant levels of Ca^{2+}-activated neutral proteases that become active when Ca^{2+} is elevated. Ca^{2+}-activated neutral proteases have been shown to cleave the RYR without greatly modifying function (59) and have been noted to be elevated after endurance-type exercise (12). However, this hypothesis was not supported, as the application of protease inhibitors did not prevent the uncoupling of ECC (29, 83). The pH and temperature dependence of these effects indicate the possible involvement of an enzymatic element such as Ca^{2+}-activated protein phosphatases and kinases.

Despite the unknown origin of these observations, the effect of Ca^{2+}-induced uncoupling may actually serve to preserve the integrity of muscle cells during extreme conditions. Reductions in ECC and Ca^{2+} release may provide an opportunity to limit releasable Ca^{2+} while sequestration continues. This would favor a lower Ca^{2+} environment and reduce Ca^{2+}-mediated muscle damage (9, 21). Elevated Ca^{2+} concentrations during fatiguing exercise need closer examination to determine if and how this event contributes to the decline in muscle performance.

Metabolism within the triadic space (microcompartment). The increase in energy production that accompanies contractile activity can significantly alter the metabolite levels of skeletal muscle. It is believed that part of the reduction in Ca^{2+} release may be of metabolic origin, since in some cases restoration of normal metabolite levels also restores some of the Ca^{2+}-activated force. The relationship between metabolites and muscle function is extremely complex. For example, reductions in pH observed during high-intensity, short-duration exercise correlate with the reduction in pH during most fatigue protocols (23, 37). However, during the ensuing recovery process, tension returns to normal more rapidly than does pH (18), and the negative effects associated with pH changes have been shown to be less significant as experimental muscle temperature approaches physiological levels (134). This uncoupling of muscle function from metabolite restoration during the recovery process suggests that muscle fatigue is multifactorial.

One reason for the inconsistency between changes in global cell metabolism and force mediated by SR Ca^{2+} release may be the architecture of the triadic region. It is possible that any number of physical constraints may prevent diffusable mediators from entering or exiting the tightly controlled space. Moreover, a microcompartment surrounding the SR Ca^{2+} channel would allow for a more precise regulation of compounds that might modulate, if not control, Ca^{2+} release. Supporting this contention are the presence of glycolytic enzymes within the triadic space that modify local ATP production (66, 142) and the existence of a microenvironment surrounding the SR Ca^{2+}-ATPase (81). Rather than address all of the possible permutations of different metabolites that would be present during a wide variety of muscle activation patterns, I will focus on a few key metabolites that have been shown to affect the SR Ca^{2+}-release process and concentrations of which are likely to be modified within the triadic space.

Glycogen. Glycogen is tightly associated with SR membranes in skeletal muscle (45, 54), and its reduction in concentration has long been identified with the onset of muscle fatigue (14). Glycogen is critical for energy production and carbon flux through glycolysis, but it is not understood how its depletion reduces muscle performance. Due to its association with the SR, glycogen has been proposed to modulate Ca^{2+} efflux from and sequestration into the SR. Several investigators have shown a reduction in Ca^{2+}-ATPase activity (13, 22, 78) following exhaustive exercise, and Byrd et al. (22) correlated this with a reduction in muscle glycogen. Using the same running protocol, Favero et al. (46) showed a depression in SR Ca^{2+} release and [^{3}H]ryanodine binding indicative of modification of the RYR channel, and Friden et al. (54) showed that intense exercise preferentially depleted muscle glycogen in the I band region, which corresponds to the terminal cisternae region of the SR. More recently, in mouse single muscle fibers, Chin and Allen (30) demon-

strated a glycogen-dependent failure of SR Ca^{2+} release. From their data, they concluded that reductions in muscle glycogen reduced the $[Ca^{2+}]_i$ and force, suggesting that glycogen availability was important for SR Ca^{2+} release. After stimulation, fibers incubated in 5.5 mM glucose resynthesized glycogen to control levels and demonstrated an enhanced fatigue resistance compared with fibers incubated in a glucose-free medium. Chin and Allen hypothesize that glycogen localized to the triadic region of skeletal muscle provides a functional coupling between ATP generated by glycolysis and that produced within the triadic gap [see Han et al. (66), ATP section]. Thus, when triadic glycogen is reduced, SR Ca^{2+} release and force production are concomitantly reduced, providing a possible communication between the current state of muscle metabolism and the membrane-protein system that regulates intracellular Ca^{2+}.

It is clear that muscle glycogen is a vital component of metabolism and may provide a functional and structural communication between the SR and the muscle cell during periods of prolonged contractile activity. Continued work to determine a direct link between glycogen and the function of the RYR channel will provide important evidence as to the mechanism by which the depletion in muscle glycogen modifies Ca^{2+} release in skeletal muscle and reduces contractile performance.

ATP. It has long been suggested that depletion of high-energy phosphates is involved in muscle fatigue (126). However, much of our current evidence does not support this contention [see Fitts (49)]. It is currently thought that bulk muscle ATP does not drop significantly before other factors that reduce force production (15). Still, ATP activates RYR channels (116), and it has been shown that ATP synthesis and consumption are microcompartmentalized in isolated skeletal muscle triads (66). The triad-synthesized ATP is not in equilibrium with the overall muscle ATP (66), suggesting that a depletion of local ATP in the junctional region could affect Ca^{2+}-release channels, the activity of which is stimulated by ATP. Allen et al. (5) have also demonstrated that caged ATP, when activated by flash photolysis in a fatigued fiber, produced a concentration-dependent increase in tetanic Ca^{2+} concentration and force. They speculated that an ATPase, the activity of which is compromised by fatigue, resides in the triadic space and is responsible for phosphorylating a protein essential for full activation of ECC (50). Until additional studies clarify the role of ATP in the ECC process as well as determine its ability to diffuse into and out of the triadic space, this issue will remain incompletely characterized.

Lactate. Lactic acid accumulation has long been considered a potential contributor to muscle fatigue, but it is now generally agreed that the reduction in tension development occurs through the effect of increased H^+ on cross-bridge formation (33, 88, 97). However, a recent report indicates that lactate can reduce tension development in working dog muscle (67). Lactate infused into arterial blood significantly increased muscle lactate concentrations without any decline in arterial or muscle pH. Although initial tension was unchanged after lactate infusion, the continued presence of lactate exacerbated the decline in tension observed during repetitive stimulation (67). Subsequently, during lactate washout of the muscle, the inhibition of tension development was alleviated. To this end, lactate (30 mM) significantly inhibits SR Ca^{2+} release by a variety of activators, including Ca^{2+}, caffeine, hydrogen peroxide, and Ag^+ (47, 48). The inhibitory effect of lactate was confirmed in [^{3}H]ryanodine-binding experiments and single Ca^{2+} channel analysis (47, 48). Lactate inhibition of Ca^{2+} channel activity should reduce the amount of Ca^{2+} released after normal muscle activation. As with Mg^{2+}, reductions in SR Ca^{2+} release mediated by lactate most likely follow the rise and fall of lactate levels and would be operative only when lactate is elevated. This is the likely case, as pretreatment of SR vesicles with 30 mM lactate failed to inhibit Ca^{2+} release when the lactate-treated SR was diluted into a lactate-free flux medium and release experiments were conducted (final lactate concentration <1 mM, unpublished observations).

Considering the evidence in the previous sections, we can infer that a pool of local metabolites may be responsible for modulating Ca^{2+} release and thus force production. Beginning with glycogen and the understanding that its reduction correlates with fatigue and ending with modifications in glycolytic intermediates, it is likely that modification of intracellular Ca^{2+} concentrations by various metabolites may influence the Ca^{2+}-release process and, ultimately, force production. This follows the notion of Williams and Klug (140), who argued that control of intracellular Ca^{2+} concentration is a critical link between muscle activity and fatigue. They suggest that alterations in intracellular Ca^{2+} handling often precede the decline in force production and thus may function by downregulating the energy cost of contractility. The concomitant reduction in energy usage and force production may be a transient trade-off in order to maintain the structural and functional integrity of the cell.

Mg^{2+}. Mg^{2+} is a potent inhibitor of the SR Ca^{2+} channel, both in isolated SR (95) and in single-fiber experiments (75). It is believed that Mg^{2+} and Ca^{2+} share and compete for common binding sites on the RYR (95, 101). Mg^{2+} concentrations rise almost twofold (from 0.86 to 1.55 mM) during tetanic stimulation of mouse muscle fibers (130). Intracellular Mg^{2+} concentration could rise as a result of reductions in ATP as Mg^{2+} binds less strongly to ADP. This possibility is difficult to assess, considering that global ATP concentration in the muscle is most likely unaltered during activity. On the other hand, as intracellular Ca^{2+} rises, it is buffered by several Ca^{2+}-binding proteins, including parvalbumin. Ca^{2+} binds to one of the two divalent cation-binding sites on parvalbumin by displacing Mg^{2+} (60). This alone may be significant, considering that parvalbumin concentrations have been reported to be as high as 0.5 mM in some species of muscle cells (60). Mg^{2+} may modify channel function during periods of contrac-

tile activity where its concentration increases. The hypothesis presented by Stephenson et al. (120) regarding the possibility that Mg^{2+} binding and debinding is responsible for Ca^{2+} channel opening supports continued research into this area. It is probable that cellular Mg^{2+} concentrations are modified as a result of prolonged contractile activity, but it remains to be established how they influence the SR Ca^{2+}-release process and muscle fatigue.

ROS. ROS have drawn attention because of their potential to disrupt normal muscle function by targeting specific proteins for modification. Molecular oxygen-derived intermediates are produced extensively in living tissues undergoing oxidative stress, such as ischemia, reperfusion, and vitamin deficiency (53). Over a decade ago, the first reports demonstrated that ROS were generated during exercise (36, 71). Since then, many reports have indicated that free radicals may play a significant role in muscle damage and fatigue that occurs with exhaustive type of exercise (see Refs. 114 and 115 for more extensive reviews).

Free radicals may be generated by several pathways in skeletal muscle (115). They include the electron transport system, located in the mitochondrial inner membrane system, and the xanthine oxidase enzyme system. The active muscle cell is a highly oxidative environment. At rest, ~2–4% of oxygen transport through the mitochondria escapes in the form of free radicals, typically superoxide. Numerous muscle cell scavenger mechanisms, such as superoxide dismutase and catalase, detoxify free radicals. Within the resting cell, the defense mechanisms far exceed the production of free radicals. However, during exercise, mitochondrial oxygen consumption can increase 20-fold, and free radical production may overwhelm the cellular defense mechanisms. Several investigators have measured free radical production as a result of contractile activity either directly or via indirect methods such as marker molecules [see Sen (114) for extensive list].

As stated in the aforementioned section, endogenous SH groups localized to the SR Ca^{2+}-release channel are thought to play a significant role in its regulation (1). It is believed that oxidation of SH groups triggers the opening of the SR RYR release channel while reduction of the disulfide bond induces closure of the channel. This theory is supported by the following observations. *1*) SR Ca^{2+} release is potently activated by oxidizing agents and the binding of heavy metals to SH groups localized to the RYR. *2*) Oxidation-induced release is sensitive to all of the known modulators of SR Ca^{2+} release; i.e., it is potentiated by adenine nucleotides, Ca^{2+}, and caffeine and inhibited by ruthenium red, tetracaine, and reducing agents (3). These results have been confirmed at the single-channel level, and whereas most activators of release are inhibited by Mg^{2+} at any concentration, SH oxidation-induced release is stimulated by concentrations up to 1 mM (physiological) and inhibited at higher Mg^{2+} concentrations (1). Whereas it is unlikely that SH oxidation is the physiological trigger for activation of SR Ca^{2+} release, our current understanding suggests that it is a viable mechanism for modifying normal ECC during oxidative stress.

The SR Ca^{2+}-release channel displays redox sensitivity (144), and exercise-induced alterations in the cell redox environment may modify its function. The redox environment of the active muscle is dependent on the GSH/GSSG ratio. Glutathione, a tripeptide, is one of the most abundant intracellular thiols found in the body. It is a scavenger of free radicals, and in the process it becomes oxidized to GSSG. Within the cytosol, the intracellular ratio of GSH to GSSG is maintained at a relatively high level (range of 5–20:1) and, in part, it determines the oxidation-reduction state in cells. During oxidative stress, such as exercise, this ratio may become altered, inactivating enzymes that bear free SH groups and result in the formation of inter- or intramolecular mixed disulfides. It has been reported that exercise significantly alters the GSH/GSSG of skeletal muscle excised from animals run to exhaustion (73). This suggests that not only is the cell redox environment perturbed, possibly by free radicals, it indicates that oxidation does alter the cell thiol status. Thus the RYR channel, which contains sensitive SH groups, appears a likely target for modification. It has been directly demonstrated that the skeletal muscle RYR channel is sensitive to singlet oxygen (122), hydrogen peroxide (48, 99) and hypochlorous acid (128). Recently, Oba et al. (99) demonstrated that hydrogen peroxide initially potentiated twitch tension. This increase was followed by a significant decline over time in single fibers. An interesting theory of ROS-muscle interactions has been proposed by Reid and co-workers (106, 108). They suggest that ROS are obligatory for normal ECC in skeletal muscle. It was shown that depletion of ROS via the addition of antioxidants depresses the twitch response and contractile function of unfatigued diaphragm muscle. Conversely, during fatiguing stimulation, the removal of ROS slows the fall of force, thus improving contractile performance.

The effects of nitric oxide (NO) on contractile function are rapidly coming into focus (119). Moreover, because NO reacts with other free radicals, it has become more difficult to evaluate the effects of ROS on in vivo muscle contraction. NO is a short-lived species that can react with molecular oxygen and superoxide anions to generate NO derivatives that can participate in redox reactions (119). Free thiol groups are the primary targets for NO modification, either by *S*-nitrosylation or by influencing disulfide formation (118). It is not surprising to find that NO can target SR Ca^{2+}-release channels. NO has been shown to inhibit channel opening (3) as well as activate the channel at higher concentrations (3, 121). This biphasic behavior is directly opposite of that observed when using hydrogen peroxide, where activation occurs at lower concentrations and inhibition of channel openings occurs at increasingly higher concentrations (48). Aghdasi et al. (3) proposed that lower concentrations of NO protect the Ca^{2+}-release channel from oxidation, whereas higher concentrations induce intermolecular cross-linking that results in an

increase in Ca^{2+} channel P_o. Physiological effects of NO on skeletal muscle and Ca^{2+} channel regulation have yet to be determined. However, considering the effects on SR Ca^{2+} channel SH groups, it remains a viable mechanism to influence Ca^{2+} regulation in muscles undergoing activity.

Despite the significant number of studies that implicate ROS in muscle fatigue and muscle damage, we have only begun to understand the relationship between ROS and muscle function in both the rested and active state. Moreover, because of the ability of NO to react with ROS, it appears that their collective activities may be linked in some manner to control and/or modify Ca^{2+} regulation in muscle cells.

CRITICAL QUESTIONS

Several areas need to be considered to provide a clearer understanding of any potential mechanism that involves reduced SR Ca^{2+} release.

1) *Proteins*. Continued evaluation of all proteins, existing and emerging, needs to focus on more precise anatomic placement, leading to a more advanced understanding of individual or complex function. A more accurate map of the triadic space should help determine the accessibility of diffusable mediators that may modify SR Ca^{2+} channel function.

2) *Multiple regulators*. Many important metabolites have been shown to alter SR Ca^{2+} channel function, but they are often evaluated independently. Our current knowledge would be greatly extended if experiments were designed to assess how multiple regulators interact with each other and with key triadic proteins.

3) *Techniques*. What new and emerging techniques will shed light on events that have yet to be described during or following intense prolonged muscle activity? For example, utilization of single-channel analysis or Ca^{2+} sparks techniques that may provide molecular insights into channel dysfunction.

Conclusions. In light of the fact that the detailed mechanism that describes ECC and SR Ca^{2+} release in muscle fibers is currently unresolved, our common understanding suggests that a variety of contractile patterns that elicit muscle fatigue appear to involve alterations in ECC and reduced SR Ca^{2+} release. Several putative mechanisms have been evaluated that represent collective ideas forwarded by many different investigators. Much work has significantly focused the discussion and areas of experimentation regarding SR Ca^{2+} release and muscle fatigue. A continued synthesis of experimental techniques, from isolated protein experiments involving single-channel analysis to controlled experiments using fiber bundles activated by an intact nerve, should provide further understanding of muscle performance observed during animal or human activity.

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