

# Skeletal muscle fibers: Inactivated or depleted after long depolarizations?

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Skeletal muscle consists of large multinucleated fibers that rapidly contract in response to action potentials generated in their surface membrane. The fiber's contractile apparatus is activated by  $\text{Ca}^{2+}$  ions that are released into the cytoplasm from an extensive intracellular storage compartment, the sarcoplasmic reticulum (SR), in response to the action potential.  $\text{Ca}^{2+}$  concentration changes in the cytoplasm during the excitation–contraction (EC) coupling process have been recorded for several decades, but only in recent years have methods been developed to monitor  $\text{Ca}^{2+}$  in the SR. In this issue of the Journal, [Robin and Allard](#) make use of this approach to study the functional basis of changes in the filling state of the SR during long-lasting membrane depolarizations.

## Voltage-controlled $\text{Ca}^{2+}$ release in muscle fibers

The efflux of  $\text{Ca}^{2+}$  from the SR is mediated by ryanodine receptors (RyRs), members of a family of giant intracellular channel proteins. The subtype 1 (RyR1) forms regular arrays in specialized zones of the SR membrane that communicate with the transverse tubules (TTs), narrow invaginations of the surface membrane, which conduct the surface action potential into the center of the fiber (Manno et al., 2013). The TT–SR junction is the basis of a unique voltage control mechanism for the rapid opening and closing of the RyR1 in response to action potential depolarization and repolarization (Melzer et al., 1995; Dulhunty, 2006; Baylor and Hollingworth, 2012). Whereas “standard” voltage-activated channels contain both of their essential functional elements, i.e., a voltage sensor and an ion-conducting pore, within a single protein, the RyR1 protein has no comparable voltage-sensitive region. The SR membrane does not seem to develop a substantial voltage, either at rest or during  $\text{Ca}^{2+}$  release (Fink and Veigel, 1996). Instead, it relies on the voltage of the TT membrane and the voltage sensitivity of a separate protein located in this membrane, the dihydropyridine receptor (DHPR; an L-type  $\text{Ca}^{2+}$  channel,  $\text{Ca}_v1.1$ ), which is mechanically connected to the RyR1. The DHPR itself is not a very effective  $\text{Ca}^{2+}$  channel. Its pore-opening reaction is much slower than the coupling reaction that bridges the junctional gap

and gates the RyR1. Moreover, opening the DHPR pore requires stronger depolarization than does the coupling reaction, and the  $\text{Ca}^{2+}$  inward current it passes is far smaller than the  $\text{Ca}^{2+}$  release flux from the SR (Ursu et al., 2005). In fact, in some fish (higher teleosts), the DHPR does not pass  $\text{Ca}^{2+}$  at all (Schredelseker et al., 2010).

The TT voltage controls the gate of the RyR1 but—being generated in a separate membrane—has no direct influence on the driving force for the efflux of  $\text{Ca}^{2+}$  from the SR, which is provided by the steep transmembrane  $\text{Ca}^{2+}$  concentration gradient between the SR lumen and the cytoplasmic space. The resting gradient is established by the constant action of adenosine triphosphate (ATP)-driven  $\text{Ca}^{2+}$  pumps (SERCA) and supported by  $\text{Ca}^{2+}$  buffers in the SR lumen, of which calsequestrin is the most important (Murphy et al., 2009). Calsequestrin's high capacity (up to 80  $\text{Ca}^{2+}$  per molecule) enables storage of large amounts of  $\text{Ca}^{2+}$  in the SR and further stabilizes the concentration gradient during  $\text{Ca}^{2+}$  release. The affinity of calsequestrin for  $\text{Ca}^{2+}$  appears to decrease when the free  $\text{Ca}^{2+}$  concentration in the SR starts to fall, and it may even act as a funnel that guides  $\text{Ca}^{2+}$  ions to the releasing sites (Royer and Ríos, 2009). Moreover, calsequestrin probably also acts as a  $\text{Ca}^{2+}$  sensor that modulates RyR permeability depending on the  $\text{Ca}^{2+}$ -loading state of the SR (Beard et al., 2009).

## Voltage-dependent inactivation of EC coupling

Loss of the normal resting membrane potential may occur in extensively stressed muscle fibers. If not counteracted by protective mechanisms, this condition would lead to maintained activation of  $\text{Ca}^{2+}$  entry from the extracellular space and  $\text{Ca}^{2+}$  release from the SR, and to dramatically increased ATP consumption. Long-lasting depolarization leads to the spontaneous relaxation of force, a phenomenon first analyzed by Hodgkin and Horowitz (1960) in frog muscle using potassium-induced contractures. They suggested that depolarization “liberates an activator which is used up in generating tension.” Later, this spontaneous relaxation process was linked to the immobilization of a transverse tubular charge

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movement (Chandler et al., 1976) and attributed to an inactivation process. During strong depolarization the transition from the activatable to the inactivated state takes seconds to develop, and it takes minutes to fully recover from inactivation after repolarization. This inactivation is enhanced by L-type  $\text{Ca}^{2+}$  channel antagonists (Berwe et al., 1987; Feldmeyer et al., 1990). Indeed, like other voltage-dependent channels, the DHPR enters an inactivated state during long-lasting membrane depolarization (Cota et al., 1984). Inactivation can be studied by recording the L-type  $\text{Ca}^{2+}$  current or the gating charge movement produced by the DHPR. Like its voltage-dependent activation, inactivation of the DHPR is communicated to the RyR1 and leads to its closure.

Availability curves, i.e., the graded sigmoidal dependence of fractional force or  $\text{Ca}^{2+}$  release activation on steady-state voltage, belong to the standard repertoire in the characterization of the EC coupling mechanism. It is generally assumed that these curves demonstrate the voltage dependence of the inactivation mechanism of the voltage sensor. Yet, in experiments in which L-type  $\text{Ca}^{2+}$  current and  $\text{Ca}^{2+}$  release are measured in parallel,  $\text{Ca}^{2+}$  release availability reaches its half-maximal value at more negative holding potentials compared with the availability of the  $\text{Ca}^{2+}$  inward current produced by the DHPR (Andronache et al., 2009). One explanation would be that the voltage control of RyR1 inactivation and the inactivation of the L-type channel permeation pathway are mediated by different states of the DHPR. A comparable situation is found in the activation process in which different states of the DHPR, attained at different voltages, appear to control  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  entry, respectively (García et al., 1994; Dietze et al., 2000). In this issue, Robin and Allard (2013) challenge the view that DHPR inactivation is the dominant cause of the decline in  $\text{Ca}^{2+}$  release during long-lasting depolarization. Instead, they suggest that  $\text{Ca}^{2+}$  depletion in the SR lumen shapes the “steady-state availability curve” of  $\text{Ca}^{2+}$  release.

#### Monitoring $\text{Ca}^{2+}$ in the storage compartment

It is obvious that the large voltage-activated flux of  $\text{Ca}^{2+}$  from the SR may also appreciably alter its own driving force. However, separating RyR gating from SR depletion is difficult. Until recently, indirect methods were used to estimate the  $\text{Ca}^{2+}$  content of the SR and its changes during various  $\text{Ca}^{2+}$  release paradigms (Pape et al., 1995; Posterino and Lamb, 2003). Within the last couple of years, progress has been made in imaging intra-SR calcium using fluorescent probes (Rudolf et al., 2006; Ziman et al., 2010; Sztretye et al., 2011; Robin and Allard, 2012). Two different methods have been established. One uses the permeable acetoxymethyl ester form of a synthetic fluorimetric dye loaded into the SR lumen. The other uses protein-based  $\text{Ca}^{2+}$  sensors that can specifically be targeted to the SR. The indicators should be of low affinity to avoid saturation by the high resting free  $\text{Ca}^{2+}$

concentration in the SR ( $\sim 0.5$  mM). The advantages and disadvantages of the two methods have recently been summarized by Manno et al. (2013). Robin and Allard (2013) used the first method in their experiments and the indicator Fluo-5N (Kabbara and Allen, 2001; Ziman et al., 2010) to record intra-SR  $\text{Ca}^{2+}$  concentration changes. They combined this with an electrophysiological technique termed “silicon clamp” (Pouvreau et al., 2007), which allows single electrode voltage clamping of large muscle cells using conventional whole cell patch clamp circuitry. The notorious problems of space-clamping muscle fibers are avoided by electrically isolating a small region of the cell by covering the rest of it with a silicon grease. The micropipette used for voltage clamping dialyses the intracellular space with an artificial solution to dilute the indicator present in the cytoplasm. In this study, the pipette solution also contained a very high concentration of the  $\text{Ca}^{2+}$  chelator EGTA (50 mM). This ensures that any residual cytoplasmic Fluo-5N produces negligible fluorescent  $\text{Ca}^{2+}$  transients during depolarization and, therefore, permits the specific recording of free  $\text{Ca}^{2+}$  concentration changes within the SR. Conditions remain remarkably stable for considerable periods of time in this configuration and thus permit the repeated fluorimetric recording of  $\text{Ca}^{2+}$  concentration changes within the SR during the long-lasting prepulse protocols that are necessary to determine the steady-state voltage dependence of  $\text{Ca}^{2+}$  release availability. Under comparable conditions, Robin and Allard (2013) also recorded cytoplasmic  $\text{Ca}^{2+}$  transients using Fura-2. Inspecting the decay of a cytoplasmic  $\text{Ca}^{2+}$  transient that occurs during maintained depolarization does not allow one to determine whether it results from inactivation of the release pathway or SR  $\text{Ca}^{2+}$  depletion. When looking at the  $\text{Ca}^{2+}$  level within the SR, however, the distinction is possible: depletion will cause a decrease, whereas inactivation will lead to the opposite. Exploiting these properties, the study came to the interesting conclusion that the slow decline in  $\text{Ca}^{2+}$  transients recorded with cytoplasmic indicators during long-lasting depolarization is largely caused by depletion. Inactivation gets started only at lower (more depolarized) membrane potential and follows with a delay.

These findings are compatible with the discrepancy between steady-state availability curves of  $\text{Ca}^{2+}$  release and L-type  $\text{Ca}^{2+}$  current we have observed in our own experiments (Ursu et al., 2004; Andronache et al., 2009). We noticed a markedly elevated free cytoplasmic  $\text{Ca}^{2+}$  concentration in a voltage range whose center was  $\sim 20$  mV more positive than the threshold for release activation. We viewed this steady increase in basal  $\text{Ca}^{2+}$  concentration as resulting from a window  $\text{Ca}^{2+}$  release (Ursu et al., 2004; Andronache et al., 2009) in analogy to the window current known from “conventional” voltage-dependent channels. Window currents arise from the overlap between activation and availability curves at voltages where

there is some degree of activation but incomplete steady-state inactivation. During long depolarizations, even a small fractional activation of the  $\text{Ca}^{2+}$  release mechanism may shift a substantial amount of  $\text{Ca}^{2+}$  from the SR lumen to the cytoplasmic space. When we depolarized the muscle fiber membrane beyond a certain value, the elevated cytoplasmic basal  $\text{Ca}^{2+}$  concentration declined to normal values. The fluorescence signal from the intra-SR  $\text{Ca}^{2+}$  sensor provides a direct demonstration of this effect in Fig. 3 A of Robin and Allard (2013) and in a previous paper by the Allard laboratory (Robin et al., 2012): When the voltage step exceeds a certain depolarization level and duration, the fluorescence signal starts rising instead of further decreasing, showing that  $\text{Ca}^{2+}$  returns into the release compartment. This is interpreted as occurring because more and more DHPR voltage sensors and consequently RyRs enter inactivated states, and release finally falls to a level that can be overcompensated by the activity of the SERCA pumps. Thus, it is likely that the shape of the steady-state availability curve of  $\text{Ca}^{2+}$  release assessed by cytoplasmic  $\text{Ca}^{2+}$  indicators does not simply reflect the voltage dependence of the DHPR-mediated inactivation gating of the RyR1 but is mixed with or even dominated by changes of the driving force for  $\text{Ca}^{2+}$  efflux from the SR as a result of depletion.

There is agreement in general but deviation in detail between the present results and our previous findings. In the present study, the voltage ranges of  $\text{Ca}^{2+}$  release leading to depletion and inactivation are apparently farther apart from each other than found in our experiments (Andronache et al., 2009). As a consequence, the predicted window range is substantially broader. This mainly seems to result from a considerably more negative voltage threshold for  $\text{Ca}^{2+}$  release activation. Because the experimental design differs, it is not evident what causes these differences. One possible reason is the buffering to very low cytoplasmic free  $\text{Ca}^{2+}$  concentrations in the present experiments because the internal solution did not contain any  $\text{Ca}^{2+}$ . On one hand this may impede reloading of the SR by the SERCA pumps and might cause a right shift in the apparent voltage dependence of DHPR inactivation, on the other hand it may interfere with  $\text{Ca}^{2+}$ -dependent inactivation (Schneider and Simon, 1988; Jong et al., 1995) and therefore lower the voltage threshold for activation.

#### Is SR depletion a player in muscle fatigue?

A further question emerging from these findings is whether SR  $\text{Ca}^{2+}$  depletion or DHPR inactivation could contribute to skeletal muscle fatigue caused by strenuous exercise. To investigate this point, Robin and Allard (2013) studied intra-SR  $\text{Ca}^{2+}$  changes during various protocols in which long trains of action potentials or voltage pulses were applied. Action potentials did in fact produce significant SR  $\text{Ca}^{2+}$  depletion but no DHPR inactivation even if applied in rapid succession. Only repetitions of

rectangular pulses that were considerably longer than an action potential produced clear evidence of inactivation, i.e., a recovery of SR  $\text{Ca}^{2+}$  after an initial decline during the course of stimulation. According to current knowledge, it is unlikely that muscle fatigue can be attributed to a single cellular event; instead, it includes contributions from action potential changes, alterations in the contractile apparatus, and  $\text{Ca}^{2+}$  release (Allen et al., 2008). Therefore, the authors' conclusion that SR depletion may predominantly contribute to fatigue should be considered with a bit of caution. Their method required intracellular conditions that are quite far from physiological. ATP turnover was probably far lower than under normal conditions in working muscle during high frequency stimulation, and the very high concentration of the  $\text{Ca}^{2+}$  chelator in the cytoplasm likely favored SR depletion. It seems worthwhile to further investigate this question using less invasive methods. Here, protein-based  $\text{Ca}^{2+}$  indicators that can be used even in intact muscle (Rudolf et al., 2006) could show their full potential.

I thank Dr. Zoita Andronache for helpful discussions.

This work was supported by Deutsche Forschungsgemeinschaft (DFG) grant ME 713/18-2

Edward N. Pugh Jr. served as editor.

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