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COMMENTARY

No voltage change at skeletal muscle SR membrane during Ca^{2+} release—just Mermaids on acid

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Calcium ions control multiple physiological functions by binding to extracellular and intracellular targets. One of the best-studied Ca^{2+}-dependent functions is contraction of smooth and striated muscle tissue, which results from Ca^{2+} ligation to calmodulin and troponin C, respectively. Ca^{2+} signaling typically involves flux of the ion across membranes via specifically gated channel proteins. Because calcium ions are charged, they possess the ability to generate changes in the respective transmembrane voltage. Ca^{2+}-dependent voltage alterations of the surface membrane are easily measured using microelectrodes. A well-known example is the characteristic plateau phase of the action potential in cardiac ventricular cells that results from the opening of voltage-gated L-type Ca^{2+} channels. Ca^{2+} ions are also released from intracellular storage compartments in many cells, but these membranes are not accessible to direct voltage recording with microelectrodes. In muscle, for example, release of Ca^{2+} from the sarcoplasmic reticulum (SR) to the myoplasm constitutes a flux that is considerably larger than the entry flux from the extracellular space. Whether this flux is accompanied by a voltage change across the SR membrane is an obvious question of mechanistic importance and has been the subject of many investigations. Because the tiny spaces enclosed by the SR membrane are inaccessible to microelectrodes, alternative methods have to be applied. In a study by Sanchez et al. (2018. *J. Gen. Physiol.* https://doi.org/10.1085/jgp.201812035) in this issue, modern confocal light microscopy and genetically encoded voltage probes targeted to the SR were applied in a new approach to search for changes in the membrane potential of the SR during Ca^{2+} release.

Ca^{2+} release in skeletal muscle

Mature muscle fibers are large, multinucleated cells. To rapidly activate the entire cross section of a fiber, the surface action potential is guided into the center of the cell by means of the extensive transverse tubular (TT) system. Within the TT membrane, L-type Ca^{2+} channels (dihydropyridine [DHP] receptors) sense the depolarization and activate Ca^{2+} release from the adjacent terminal cisternae of the SR. The flux of Ca^{2+} passed by the L-type channels is much smaller than the release flux (Brum et al., 1987; Ursu et al., 2005), and, unlike in cardiac cells, it is not required for release activation. Instead, the information is transmitted across the junctional gap separating TT and SR membranes by conformational coupling between the DHP receptor and the Ca^{2+} release channel (ryanodine receptor [RyR]; Bannister, 2016).

Like transmembrane voltage, the Ca^{2+} current through SR RyRs that results from TT membrane depolarization cannot be measured directly. Instead, it can be calculated (when other sources are negligible) from the time course of the increase in Ca^{2+} concentration in the myoplasm derived from an indicator dye (Baylor et al., 1983; Melzer et al., 1987; Pizarro and Ríos, 2004). The calculation involves estimates of the amounts of Ca^{2+} bound to intracellular sites and removed by active transport. There is general agreement that the net flux of Ca^{2+} to the myoplasm during rapidly repeating action potentials or during voltage steps lasting fractions of a second is large initially but falls rapidly to a value severalfold smaller. These kinetic characteristics have been attributed mainly to the gating of RyRs in response to a combination of dihydropyridines receptor (DHPR) voltage activation and positive and negative feedback from the released Ca^{2+}. Positive feedback (Ca^{2+}-induced Ca^{2+} release) seems to play a minor role in mammalian skeletal muscle but may emerge under some pathological conditions (Ríos, 2018). Negative feedback (Ca^{2+}-dependent inactivation) is a characteristic common to mammalian and nonmammalian muscle.

In addition to RyR gating, changes in the chemical and electrical driving forces for Ca^{2+} would contribute to the time course of Ca^{2+} release. Because each calcium ion carries two positive charges, the release flux of Ca^{2+} might lead to the buildup of a negative potential on the luminal side of the SR. Fluxes of other ions are necessary to balance at least part of the charge to permit release to continue for the observed intervals of time (Fink and Veigel, 1996; Takeshima et al., 2015). If no such balance took place,

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the Ca^{2+} equilibrium potential would be reached and rapidly prevent any further release. In addition, Ca^{2+} release changes the concentration gradient and Nernst equilibrium potential of Ca^{2+} at the SR membrane. Both effects might contribute to the characteristic phasic time course of the Ca^{2+} release flux. It is, therefore, of great mechanistic value to obtain reliable information on these changes. The study by Sanchez et al. (in this issue) reaches an important milestone by presenting the first convincing measurements of the SR membrane voltage during Ca^{2+} release.

Counter ion pathways of the SR membrane

First clues about the putative size of the SR voltage came from electron-probe x-ray microanalysis of ultrathin muscle cryosections (Somlyo et al., 1981, 1985). Because of the effective action of the SR/ER Ca^{2+}-ATPase (SERCA) Ca^{2+} pump, a free Ca^{2+} gradient of up to 10^5 is achieved across the SR membrane in the steady state at rest. In spite of the very large concentration difference, estimates of the concentrations of several other ion species (K^+, Na^+, and Cl^-) in the myoplasm and inside the SR reveal that there are essentially no gradients. This suggests that the monovalent ion permeabilities are high enough (relative to the low Ca^{2+} permeability at rest) to effectively shortcut the buildup of any resting potential across the SR membrane. The uncertainties about the absolute values of the permeabilities and the limited time resolution to measure fast ion gradient changes mean that it is essentially impossible to predict from x-ray microanalysis data whether a membrane potential change would occur during physiological Ca^{2+} release in the course of normal activity. After a tetanus lasting 1.2 s, Somlyo et al. (1981) found a 59% decrease in the Ca^{2+} content in the terminal cisternae. The corresponding release to the myoplasm was partially balanced by K^+ and Mg^{2+} entering the SR, but there remained a large unexplained charge difference. Protons (which could not be recorded with this technique) were suggested as possible candidates to balance this charge. Because the Cl^- gradient did not change in spite of the presence of Cl^- channels in the SR, it was argued that tetanic Ca^{2+} release is not associated with large or sustained changes in the SR membrane potential. In agreement with this, Baylor et al. (1984) calculated a peak SR voltage change of only 2 mV using their own determination of the Ca^{2+} release flux in single muscle fibers and an estimate of the K^+ conductance in SR vesicles.

Since the initial identification of cation and anion conductances in the SR membrane (Miller, 1978), progress has been made in further specifying the ionic pathways. However, their purposes and relative contributions are still a matter of debate (Takeshima et al., 2015). In addition to a variety of anion channels, the SR membrane contains different types of cation channels, some of them unselective. The K^+ conductance originally described is now attributed to two members of the trimeric intracellular cation (TRIC) channel family, TRIC-A and TRIC-B. Double-knockout mice lacking both TRIC channel types suffer from embryonic heart failure resulting from defective SR Ca^{2+} handling. It was therefore concluded that these channels are important for providing counter currents to maintain normal SR Ca^{2+} fluxes (Venturi et al., 2013). Their function was envisaged as follows: At rest, the majority of RyRs are closed, and protons are extruded in exchange for Ca^{2+} by the SERCA pump (Inesi and Tadini-Buoninsegni, 2014). H^+ reenters the SR lumen via a potassium proton exchanger, and a small number of open TRIC channels permits the return of K^+ to the SR and ensure equal concentration inside and outside the SR. Because of their characteristic voltage dependence, more TRIC channels will open as soon as the lumen hyperpolarizes during Ca^{2+} release and therefore provide an effective mechanism to counteract the formation of an electrical force opposing Ca^{2+} efflux from the SR.

The importance of K^+ and Cl^- channels during Ca^{2+} release was questioned by Gillespie and Fill (2008), who suggested that the release channel itself is sufficient to provide the necessary counter-ion flux for Ca^{2+} (carried by K^+ and Mg^{2+}). The passage of Cs^+ through RyR channels (but not K^+ channels) would also explain why replacement of internal K^+ by Cs^+, as done in many experiments, did not block Ca^{2+} release. This view has recently been modified in a model that incorporates non-RyR pathways for counter-ion flow in cardiac SR, which may also be applicable to skeletal muscle (Zsolnay et al., 2018). In their study, a cascading network of countercurrents is suggested in which the K^+ channels dominate, especially at rest, whereas Cl^- channels and RyRs contribute relatively little. During activated Ca^{2+} release, the relative contribution of RyR-mediated countercurrent increases. If one of the monovalent-selective channels is blocked (for instance by Cs^+), the other pathways would take over and always provide sufficient charge-compensating current for Ca^{2+} release to continue.

Membrane potential imaging using optical indicators

The indirect evidence summarized above predicts at most small changes in the SR membrane potential during Ca^{2+} flux. To firmly establish whether the identified ionic pathways indeed suppress a substantial voltage alteration upon Ca^{2+} release and reuptake, more direct measurements are required. Noninvasive optical recording is the most convenient approach. Any polar substance whose distribution or rearrangement caused by the transmembrane electrical field elicits an optical signal might be used as an indicator for voltage imaging. For recordings of fast membrane potential changes like neural action potentials, a sensor mechanism with minimal spatial redistribution of the optical probe is required.

Early work on frog muscle fibers reported intrinsic birefringent signals and Nile Blue A fluorescence changes, possibly resulting from a voltage change at the SR membrane, and modeled the extent to which SR membrane conductance and capacitance would influence the size and time course of a voltage change (Vergara et al., 1978; Baylor et al., 1984). However, the actual origin of these signals was uncertain, and calibration was not possible. Since early work in the 1970s, considerable progress has been made in developing optical voltage probes with improved signal/noise ratio, speed, and sensitivity (Kulkarni and Miller, 2017). Small molecule indicators like the frequently used ANNEPS dyes were primarily developed for noninvasive recording of rapid electrical activity in the outer membranes of excitable cells (Miller, 2016). A nice example of voltage imaging in muscle is the measurement of action potential propagation within the TT system using di-8-ANNEPS by shifted excitation and emission ratioing (SEER), an improved confocal microscopy technique (Manno et al., 2013).

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However, recording from an intracellular organelle like the SR poses additional challenges. In this circumstance, a genetically encoded indicator is almost indispensable because it can be directed to the desired location by means of a specific targeting sequence. A preferred indicator mechanism in protein-based probes is FRET between a donor and an acceptor that move relative to each other when driven by membrane voltage. FRET probes fused with a voltage-sensing domain derived from voltage-dependent proteins make useful indicators to detect membrane potential changes (Platisa and Pieribone, 2018). A voltage-dependent protein exploited for the design of genetically encoded membrane potential indicators is the *Ciona intestinalis* voltage sensor–containing phosphatase (Ci-VSP), an ascidian enzyme that catalyzes phosphoinositide turnover in the sperm flagella of the sea squirt and whose activity is directly regulated by membrane potential (Murata et al., 2005). Using the voltage-sensing domain of Ci-VSP, Tsutsui et al. (2008) designed a protein-based voltage indicator called Mermaid that contains sequences of two fluorescent proteins originating from corals as FRET donors and acceptors.

Voltage recording from skeletal muscle SR

In their attempt to record voltage signals from the SR membrane, Sanchez et al. (2018) used various Mermaid derivatives. Fusion with a sequence (T-301) of triadin, a protein expressed only in the junctional face of the terminal cisternae, ensured specific SR retention that was confirmed by confocal imaging. Point mutations in the voltage-sensing domain adjusted the voltage sensitivity to match the putative resting SR voltage of ∼0 mV. The cDNA was delivered to the small paw muscle cells of anesthetized mice using an in vivo electroporation procedure (DiFranco et al., 2009) that led to a patchy cellular expression of the fluorescent proteins within a few days. Electrophysiology was then performed on enzymatically isolated muscle cells using a technique developed years ago in the same laboratory called silicone clamp. This is a continuous single-electrode voltage clamp method in which most of the muscle fiber surface is covered by silicone grease to ensure reliable space clamping in the small grease-free region (Jacquemond, 1997).

Mermaid constructs lacking the SR targeting signal showed membrane expression in both the SR and TT system. Because the voltage of the external membrane (including TT system) could be electrically recorded and rapidly controlled, these constructs could be used to calibrate voltage-dependent FRET responses at a holding potential of 0 mV when the DHPR voltage sensor for Ca^{2+} release is inactivated. The rectangular voltage steps produced almost rectangular FRET signals, whose amplitudes showed saturation when plotted against voltage (resulting from the saturable displacement of the indicator's sensor charges) and could be well fitted by conventional Boltzmann functions. Restoring Ca^{2+} release by setting the holding potential to −80 mV changed the shape of the depolarization-induced FRET signals: a slower response was superimposed on the fast signals. Moreover, the specifically SR-targeted FRET indicator showed only the slow signals, a result one would expect from insufficient charge compensation during SR Ca^{2+} fluxes.

One can imagine the excitement of the authors to find a signal that, contrary to current belief, indicated a quite dramatic change in the SR membrane potential. This, at least, was the first tentative interpretation of the findings (Sanchez, C., C. Berthier, B. Allard, J. Perrot, C. Bouvard, H. Tsutsui, Y. Okamura, and V. Jacquemond. 2017. 45th European Muscle Conference in Montpellier, France. Abstr. S13.P8-211). However, the case proved to be more complicated and is an object lesson in the value of questioning intuitive explanations by more thorough experiments.

When Ca^{2+} is released into the cytoplasm, the chemical environment of the indicator changes. What if this change affects the properties of the indicator and produces an optical artifact? For various practical reasons (for instance, to suppress contraction), muscle fibers are often dialyzed with an internal solution containing millimolar concentrations of the chelator EGTA, as was the case in these experiments. EGTA releases two protons for every Ca^{2+} bound, an effect that has even been exploited to measure Ca^{2+} release with a pH-indicating dye (Pape et al., 1995). Replacing EGTA with BAPTA, a chelator that binds Ca^{2+} ions without exchanging them for protons, essentially eliminated the slow SR-specific FRET signal. In another set of experiments, modifying the pH in the myoplasm revealed a relatively strong effect of the proton concentration on steady-state fluorescence of the SR-specific Mermaid indicator, suggesting that the presumed SR voltage signal was actually an artifact resulting from a local decline in pH. This was surprising because Mermaid was originally introduced as a probe that exhibited minimal pH sensitivity (Tsutsui et al., 2008).

Released Ca^{2+} itself might also affect the indicator and cause an artifactual signal. BAPTA differs from EGTA not only in its lack of proton release when Ca^{2+} is bound but also in its speed of Ca^{2+} binding—Ca^{2+} ligation by BAPTA is much faster than by EGTA. As a consequence, the local rise in Ca^{2+} near the release channel will be much smaller and extend a shorter distance into the myoplasm from the channel pore (Pape et al., 1995). To address the likelihood of Ca^{2+} sensing by the indicator, Sanchez et al. (2018) performed similar experiments in the absence of any high internal Ca^{2+} buffering. Muscle cells would heavily contract under these conditions. Therefore, BTS, a blocker of the motor protein myosin, was applied. Again, even though depolarization-activated Ca^{2+} transients should be larger and proton transients smaller than in the original experimental setting, the FRET signal of the voltage probe in the SR membrane was very small. A small drawback, especially with regard to the last point, is the quite sparse documentation of the actual Ca^{2+} signals under the different conditions.

In summary, Sanchez et al. (2018) indicate that only minor FRET changes can be detected from SR-targeted Mermaid voltage probes when inducing large SR Ca^{2+} fluxes provided precautions are taken to avoid local changes in pH. Consequently, these experiments underpin the notion that negligible voltage changes occur at the SR membrane. They lend independent support to the hypothesis that the transfer of positive charge through the SR membrane carried by Ca^{2+} can normally be compensated by the available counter-ion conductances. For future experiments, it seems worthwhile to try to eliminate the still disturbing pH dependence of the Mermaid indicators. In combination with Ca^{2+} release flux quantification, they could then be used to reinvestigate whether alterations in the SR membrane potential are evoked by experimentally reducing counter-ion flow.

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