

Commentary

A New View of Ca²⁺ Sparks in Frog Skeletal Muscle

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Calcium sparks are brief, highly localized elevations of [Ca²⁺], which arise spontaneously or can be evoked by depolarization in many types of muscle cells. The Ca²⁺ is released into the cytoplasm from intracellular stores (the SR) through the SR Ca²⁺ release channels (the RYR). Since their discovery (in cardiac muscle; Cheng et al., 1993), Ca²⁺ sparks have attracted substantial attention. The reasons for this are clear: first, Ca²⁺ sparks almost certainly serve as “building blocks” for larger cellular Ca²⁺ signals, such as Ca²⁺ waves or the Ca²⁺ transients that trigger muscle contraction. In some cases, modulation of the whole-cell Ca²⁺ transient occurs by variation in the number of building blocks. Second, the highly localized nature of the changes in [Ca²⁺] during Ca²⁺ sparks can be important, as when Ca²⁺ sparks activate only nearby Ca²⁺-dependent K⁺ channels in smooth muscle to cause hyperpolarization and, thus, signal for muscle relaxation (Nelson et al., 1995). Finally, and this may be the most powerful attraction of Ca²⁺ sparks; they seem to offer a unique view into the molecular operation of the RYR themselves, as these are intracellular channels that are inaccessible to ordinary techniques (patch clamp). Nevertheless, Ca²⁺ sparks have proved remarkably recalcitrant to our efforts to obtain a thorough understanding of them. Some reasons for this also, are clear: first, our ability to resolve them optically is marginal, even with the best confocal microscopes. Second, a Ca²⁺ indicator is needed to make them visible, and the available fluorescent Ca²⁺ indicators are far from ideal; they bind to molecules other than Ca²⁺ (proteins) and exhibit altered properties in cells. Finally, the spatial spread of the Ca²⁺ spark is strongly influenced by a large number of factors, many of which are not well characterized quantitatively and which are not related to the molecular operation of the RYR (but rather to Ca²⁺ diffusion in a strongly buffered environment, for example). These issues, and others, come to the fore in a provocative paper published in this issue of *The Journal*. Here, Hollingworth and his colleagues (2001) provide the first detailed report on the characteristics of calcium sparks in intact frog skeletal muscle fibers (most previous studies have used “cut” muscle fibers). There are several surprises in this most recent study. Voltage-activated Ca²⁺ sparks in intact fibers decay faster, don’t spread as far in the cell, and have considerably less “mass” than their cousins in cut muscle fibers. Perhaps most impor-

tant though, the results of this very careful study in intact fibers fail to confirm some of the more exciting recent observations on Ca²⁺ sparks in cut fibers: the existence of signals postulated to arise directly from the activation of a single voltage-gated RYR. Ultimately, these issues can be clarified only by further experimentation. In the paragraphs below, I attempt to elucidate some of these issues by summarizing briefly Hollingworth et al. (2001) and comparing its results to earlier works.

The Experimental Preparation

Skeletal muscle physiologists have long used cut muscle fibers (Hille and Campbell, 1976) in their studies of excitation-contraction coupling. The cut fiber preparation is, as the name implies, a skeletal muscle fiber cut at both ends and mounted in such a way that the membrane potential of the central portion (the one studied) can be controlled. This preparation also provides the means to control the intracellular milieu and to introduce Ca²⁺ indicators, such as fluo-3. Of course, cut fibers are subject to the problem that intracellular constituents may diffuse out into the end pools if not added in appropriate amounts to the experimental solutions. Other changes, such as in fiber hydration, also may occur. In the present study, *Rana pipiens* single, intact fibers were loaded with Ca²⁺ indicator (fluo-3) by pressure microinjection during relatively brief (i.e., <3 min) microelectrode impalements of the fibers. This should be the minimally perturbing way to get a membrane impermeant form of fluo-3 into the fiber. To depolarize the fibers and observe voltage-activated (rather than spontaneous) Ca²⁺ sparks, the intact fibers were exposed to solutions high in KCl (13 mM). For measuring Ca²⁺ sparks, the optical performance of the microscope is important, and the homemade confocal microscope built by Hollingworth et al. (2001) has superior performance (i.e., spatial resolution that is close to the theoretical limit, high efficiency, and photometric accuracy). This is not likely to account for the differences between this study and the earlier ones, however; the essential difference is probably the use of intact, as opposed to cut, fibers.

[Ca²⁺]_i and Pattern of Fluorescence in Resting Fibers

Hollingworth and colleagues (2001) began by analyzing extensively the pattern of fluorescence in fibers at rest.

This might seem superfluous, but the results are significant. The very existence of periodic patterns of fluorescence in resting striated muscle most likely means that a substantial fraction of the Ca^{2+} indicator is bound and/or that bound Ca^{2+} indicator has different properties than free. As might be predicted therefore (but had not been shown before), the pattern itself was found to be Ca^{2+} -dependent, in that (presumed) elevations of internal $[\text{Ca}^{2+}]_i$ by depolarization with KCl changed the pattern. The pattern became similar to that normally reported in cut fibers, at rest. It is usually assumed that this pattern of background fluorescence is “ratioed out” when $\Delta F/F$ is calculated. If the background fluorescence were Ca^{2+} -dependent, however, as would appear from the present results, the possibility of subtle artifacts in $\Delta F/F$ is raised, if the fluorescence background signals are not treated very carefully. Also from this and other evidence, it appears that the $[\text{Ca}^{2+}]_i$ in intact fibers at rest may be as little as half the level usually set (by experimental manipulation) in cut fibers (100 nM). Confocal scanning itself also elevated the estimated $[\text{Ca}^{2+}]_i$ significantly, from 30–45 nM to 75–105 nM. The significance of a higher resting $[\text{Ca}^{2+}]_i$ in cut fibers compared with intact ones is not known, but one consequence could be a greater propensity for Ca^{2+} -induced Ca^{2+} -release (CICR) during sparks of cut fibers.

Spark Morphology

As mentioned above, Ca^{2+} sparks in intact fibers have a shorter duration, smaller spatial spread, and a much smaller mass than Ca^{2+} sparks reported by others in cut fibers (see Table VII of Hollingworth et al., 2001). The rise times were similar, however. Are these differences significant for our understanding of the molecular machinery of excitation-contraction coupling? The answer to this is not clear because the meaning of these parameters of spark morphology is not yet fully understood. With respect to rise times, a conservative explanation is that the similarity of rise times suggests that one or more RYR are open for similar periods of time in the sparks of cut and intact fibers. (The arguments and issues relating to the number of SR Ca^{2+} release channels involved in generating a spark have been summarized in recent *Perspectives* in this *Journal* [Cannell and Soeller, 1999; Schneider, 1999; Shirokova et al., 1999]). However, beyond this point, there are more differences than similarities. Almost all investigators of sparks have noted (Izu et al., 2001) that experimentally recorded Ca^{2+} sparks are larger, in spatial spread (full width at half maximum [FWHM]), than can be accounted for in mathematical models of Ca^{2+} sparks (Rios et al., 1999; Jiang et al., 1999; Izu et al., 2001). For example, Jiang et al. (1999) using an RYR channel current of 1.4 pA, produced sparks that matched experimental ones in all respects except that their FWHM was 1.0 μm , in-

stead of $\sim 2.0 \mu\text{m}$, as typically recorded in cut muscle fibers. Interestingly, the Ca^{2+} sparks in intact fibers have a FWHM of 1.0 μm . There is no single parameter of spark morphology that can be related unequivocally to the current strength; but, the present results raise the possibility that estimates of the number of RYR involved in sparks, as they occur in intact muscle, would have to be revised downward (from present estimates in the several tens of channels). In addition to the usual spark morphological parameters of FWHM, full duration at half maximum (FDHM), and peak fluorescence ratio amplitude ($\Delta F/F$), Hollingworth et al. (2001) measured a less common parameter, “spark mass” first introduced by Sun et al. (1998). According to Hollingworth et al. (2001), the signal mass “closely approximates the total increase in Ca-bound fluo-3.” Their results indicate that voltage-activated spark mass in intact fibers is about one third of that in cut fibers and as little as one tenth of that in permanently depolarized cut fibers. As in most issues with Ca^{2+} sparks, however, this finding does not have an unequivocal meaning. The small mass could result from less Ca^{2+} release, or from a loss of Ca^{2+} buffering power in cut fibers.

Ca^{2+} “Embers” and “Ridges”

An earlier report in *The Journal* (Gonzalez et al., 2000) raised the provocative possibility that certain features (“ridges” and “embers”) of voltage-activated Ca^{2+} sparks in cut muscle fibers reflect direct channel openings by the voltage sensor of excitation-contraction coupling. The ember is a late elevation of Ca^{2+} , $\sim 1.0 \mu\text{m}$ in width that continued for ~ 100 ms after the spark. A ridge of elevated fluorescence preceded voltage-activated sparks, and it was suggested that this reflected the initial opening of one RYR by a voltage sensor. The ember was thought to represent the flux of Ca^{2+} from the voltage-gated RYR, which continued for some time during the depolarization. Ridge and ember became more visible in the presence of promoters of CICR (caffeine and low $[\text{Mg}^{2+}]_i$) and less visible under conditions when CICR would be depressed (high $[\text{Mg}^{2+}]_i$). Thus, voltage-activated sparks in cut fibers in high $[\text{Mg}^{2+}]_i$ were also relatively narrow (FWHM, 0.8 μm), with prominent ridge and ember, which was interpreted to reflect a reduced number of RYRs being activated by CICR. It seems likely that voltage-activated Ca^{2+} sparks in intact fibers also involve fewer channels than sparks in cut fibers, given their smaller FWHM and strikingly smaller signal mass. Disappointingly, careful examination of the voltage-activated sparks in intact fibers failed to reveal any suggestion of the ridge, and only a very small signal that might correspond to the ember. The significance of this “failure” remains to be discerned.

Conclusions

In summary, we are left with the fact that Ca^{2+} sparks in intact fibers seem to be different in important respects from those routinely recorded in cut muscle fibers. The differences are in features of sparks that relate to our basic understanding of excitation-contraction coupling; the number of RYRs involved, how they are activated and for how long, and how much current they carry. Of course, an experimental preparation need not be "physiological" or intact to yield important information; indeed, the most informative experiments are often precisely those in which the cellular machinery is dismantled or altered significantly to reveal underlying mechanisms. The ultimate question for physiologists is always how such mechanisms really work in intact cells and tissues.

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