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Calcium-induced release of calcium in muscle: 50 years of work and the emerging consensus

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Ryanodine-sensitive intracellular Ca^{2+} channels (RyRs) open upon binding Ca^{2+} at cytosolic-facing sites. This results in concerted, self-reinforcing opening of RyRs clustered in specialized regions on the membranes of Ca^{2+} storage organelles (endoplasmic reticulum and sarcoplasmic reticulum), a process that produces Ca^{2+}-induced Ca^{2+} release (CICR). The process is optimized to achieve large but brief and localized increases in cytosolic Ca^{2+} concentration, a feature now believed to be critical for encoding the multiplicity of signals conveyed by this ion. In this paper, I trace the path of research that led to a consensus on the physiological significance of CICR in skeletal muscle, beginning with its discovery. I focus on the approaches that were developed to quantify the contribution of CICR to the Ca^{2+} increase that results in contraction, as opposed to the flux activated directly by membrane depolarization (depolarization-induced Ca^{2+} release [DICR]). Although the emerging consensus is that CICR plays an important role alongside DICR in most taxa, its contribution in most mammalian muscles appears to be limited to embryogenesis. Finally, I survey the relevance of CICR, confirmed or plausible, to pathogenesis as well as the multiple questions about activation of release channels that remain unanswered after 50 years.

Introduction

CICR is a process that occurs in many cells and tissues whereby an increase of $[Ca^{2+}]$ in the cytosol causes a further increase as Ca^{2+} is released from intracellular stores. It is the consequence at the cellular level of the ability of intracellular Ca^{2+} channels to be "activated by Ca^{2+}", that is, to open when cytosolic $[Ca^{2+}]$ rises above a threshold level.

General descriptions of Ca^{2+}-dependent processes in biology usually start by stating that Ca^{2+} is a ubiquitous cellular messenger, if not the most ubiquitous one. It is also recognized that the existence, and often coexistence, of multiple "signals" conveyed by Ca^{2+} is made possible by their encoding in specific temporal sequences and spatial scales. The encoding relies crucially on the ability of cells to increase cytosolic $[Ca^{2+}]$ to values two or three orders of magnitude above resting levels, producing events that may be quite localized (of submicron spatial spread at peak amplitude) and temporally brief, events for which Ca^{2+} sparks (Cheng and Lederer, 2008) are a paradigm.

Two properties of intracellular Ca^{2+} channels make these key features possible: the arrangement of the channels in clusters and the control of their gating by Ca^{2+} itself. Thus appointed, RyR channels can open concertedly to produce large local transients. Ca^{2+}-induced channel opening is therefore a defining property at the core of Ca^{2+} signaling.

Like many aspects of Ca^{2+} signaling, CICR was discovered and first studied in detail in skeletal muscle (Endo et al., 1970; Ford and Podolsky, 1970). As befits a fundamental process, it is now known to occur in many tissues and serve multiple purposes. In addition to the original discovery, and perhaps because of that head start, the studies of CICR in skeletal muscle went on to provide additional findings and advances, which later extended to other cells and tissues.

Shortly after the discovery of CICR in skeletal muscle, a similar process was demonstrated in cardiac muscle (Fabiato and Fabiato, 1972; Fabiato et al., 1972). CICR was later shown to be the sole mechanism of activation of Ca^{2+} release in the heart; consequently, the studies in cardiac muscle now vastly surpass, in volume and detail, what has been done in skeletal muscle.

By comparison, the relevance of CICR to skeletal muscle has always been controversial. This article reviews the research and the contested evidence for its physiological role. The studies are reviewed in a loose chronological sequence. Because the methods evolved from largely biophysical techniques in the 1970s and

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1980s to studies of structure and heterologous expression in live animals in the last decade of the twentieth century, the chronological approach allows separate consideration of the different methodologies. This article omits much to dwell on works that, in my view, provided unique mechanistic insights.

CICR in the heart has been reviewed often, with standout articles by Bers (2001) and Cheng and Lederer (2008). For this reason, the studies of cardiac muscle are examined only in cases in which the advances were first made there or where differences between the tissues provide additional insight. Direct precedents for skeletal muscle include an authoritative article by Endo (2009) and an informative examination by Murayama and Kurebayashi (2011) of the differential involvement of two isoforms of the ryanodine-sensitive ion channel, RyR, in CICR. Recently, Meissner (2017) reviewed structure–function relationships in the RyR, and Franzini-Armstrong (2018) sketched the history of the excitation–contraction (EC) coupling field.

Before the discovery of CICR

The initial observations of CICR occurred in the conceptual hotbed generated by the discovery of the role of Ca^{2+} in the control of interactions between myofilament proteins.[1] This precedent required the introduction of Ca^{2+} buffers as essential tools that would later be applied for various purposes in the study of CICR. In brief, Bozler (1954) first reported the relaxing effect of Ca^{2+} buffers on permeabilized muscle, noting their similarity with the effects of the "relaxing factor" (Marsh, 1951; Bendall, 1953) later identified with the SR. The essential role of Ca^{2+}, which justified the effect, was established by Weber (1959) and Setsuro Ebashi, who was a postdoc at that time in the Kumagai laboratory of the University of Tokyo. In a study done in 1958 that was initially confusing for its authors, Ebashi et al. (1960) found that EDTA was much less relaxing than a buffer of similar calcium affinity then called GED TA. Later, Ebashi (1960) explained this as being the result of the occupancy of EDTA by Mg^{2+}. Thus introduced, GEDTA, or EGTA as it was later named, became essential in studies that required setting Ca^{2+} at low values in the presence of much greater Mg^{2+}.

Discovery

The first demonstrations of CICR were communicated nearly simultaneously by Endo et al. (1970) working at the University of Tokyo and by Ford and Podolsky (1970) in the laboratories of the National Institutes of Health in Maryland. As narrated by Makoto Endo, their finding was the result of serendipity and scholarly duty. As reluctant Department of Pharmacology members who were really interested in physiology, Endo and starting postdoc Yasuo Ogawa were tasked to prepare something to present, "as a show of fidelity," for the 1966 meeting of the Japanese Pharmacological Society. Looking for something easy to do, they set out to confirm, in the more physiological setting of the skinned frog muscle fiber, the observation by Weber (1968) and Weber and Herz (1968), communicated preliminarily in 1965, that caffeine caused Ca^{2+} release from isolated SR. Surprisingly, they found that low caffeine induced a large contraction, which recurred at intervals of minutes. The contractions were, in fact, propagated waves of shortening moving along the fiber, which suggested a self-sustained process that could only be ascribed to activation by Ca^{2+}.

Meanwhile, in Maryland, Ford and Podolsky reached the same conclusion by directly applying high $[Ca^{2+}]$ solutions to skinned frog muscle fibers.[2] The two lines of work intersected in 1968 as back-to-back talks given by Ford and Endo at the 24th International Congress of Pharmacology held in Washington, D.C. After both presentations, Podolsky stood up to declare his "pleasure that the same discovery was done simultaneously on both sides of the Pacific Ocean" (as told by Ogawa and Endo).

The dominant mechanism of activation of Ca^{2+} release in skeletal muscle was later shown to be a conformational signal (Schneider and Chandler, 1973) induced in the L-type Ca^{2+} channel ($Ca_V1.1$ or dihydropyridine receptor [DHPR]) by depolarization of the t tubule membrane, in a process dubbed DICR (depolarization-induced Ca^{2+} release; Ríos and Brum, 1987; Tanabe et al., 1988; Nakai et al., 1996). In part as a consequence of the establishment of this mechanism as primary, the relevance of CICR to skeletal muscle was controversial from the start, with one of its discoverers as the main questioner (Endo, 2009).

The RyR in subcellular preparations

Since the identification of the SR Ca^{2+} release channels, their Ca^{2+}-dependent properties have been defined in subcellular preparations. As reviewed by Murayama and Kurebayashi (2011), a similar dependence of P_{open} (or other measures of channel openness) versus $[Ca^{2+}]$ is found in skinned frog myofibers, SR vesicles, and purified channels in bilayers. In simplified systems, including vesicles and purified proteins, [^{3}H]ryanodine binding can provide a proportional measure of channel openness.[3] Under standard near-physiological conditions, P_{open} increases with $[Ca^{2+}]$ in the micromolar range and decreases at greater concentrations. The biphasic dependence is conventionally explained by the existence of an excitatory binding site ("A" in the nomenclature of Laver et al., 1997) and an inhibitory ("I") site of much lower affinity. The fraction of activated channels (which in a statistical ensemble equals P_{open}) is

$$F_a = F_{a,max} f_A (1 - f_I). \quad (1)$$

f_A and f_I represent the fractional occupancies of the A and I sites. The observed inhibitory effects of Mg^{2+} are well described, assuming that it binds to both A and I sites; the occupancy of I by Mg^{2+} or Ca^{2+} is inhibitory, whereas that of A by Mg^{2+} inhibits by competition with Ca^{2+}. $F_{a,max}$in Eq. 1 adopts values between 0 and 1, representing the effects on the channel of factors (say, ATP) that alter its P_{open} without affecting its Ca^{2+} or Mg^{2+} sensitivity. The site occupancy is approximately described by a conventional "Hill" equation,

$$f_i = \frac{[Ca^+]^{n_{i,Ca}}}{[Ca^{2+}]^{n_{i,Ca}} + K_{i,Ca}^{n_{i,Ca}}\left(1 + [Mg^{2+}]^{n_{i,Mg}}/K_{i,Mg}^{n_{i,Mg}}\right)}, \quad (2)$$

[1] Hill (1949) had envisioned Ca^{2+} as a hypothetical activator in his argument for the inability of diffusion to effect the internal spread of activation in muscle.

[2] Ford and Podolsky also obtained contractures upon applying changes in the anion composition of the bathing solution, a result that was incorrectly interpreted for a long time as a consequence of SR membrane depolarization and only clarified two decades later by Sue Donaldson in our department at Rush University (Donaldson, 1985; Donaldson et al., 1989).

[3] Channel activity is determined using a concentration of [^{3}H]ryanodine below the K_D of its reaction with RyR, using a [RyR] that is less than [ryanodine]. Under those conditions, P_{open} is usually proportional to RyR-bound [^{3}H]ryanodine (Meissner, G., personal communication).

where index *i* represents A or I. Fig. 1 A shows experimental values and/or fits with Eq. 1 for all isoforms of the channel in the absence or presence of Mg^{2+}.

The basic properties revealed by bilayer reconstitution cannot be simply extrapolated to the living cell because the channels are sensitive to several ligands, including proteins, small peptides, and ions, as well as chemical modification (Fill and Copello, 2002; Meissner, 2017). Additionally, the independence of sites A and I implicit in Eq. 1 ignores dynamic aspects of control that determine the response in vivo. A demonstration of these complexities is in the study by Sánchez et al. (2003), which defined the $[Ca^{2+}]$ dependence of Ca^{2+} release from cardiac SR vesicles by stopped-flow mixing. Their results still show a biphasic dependence of the rate of Ca^{2+} release on $[Ca^{2+}]$ (Fig. 1 B, dashed line), but with shifts to lower values in the effective K_Ds of both activation and inactivation by nearly two orders of magnitude. Activation by submicromolar cytosolic Ca^{2+} was confirmed much later in permeabilized frog myofibers (Figueroa et al., 2012).

Many ligands other than Ca^{2+} favor the open state of the RyR channels. Three have been intensively studied and used as tools: ryanodine, because its fractional binding is used as a measure of the channel's P_{open}; ATP, because it operates as an essential cofactor that must be present for CICR to work; and caffeine, the paradigmatic promoter of activation by Ca^{2+}. In recent years, with the advent of direct electron detection cryo–electron microscopy (EM; Li et al., 2013), the structure of RyRs 1 and 2 have been determined at near-atomic resolution (Efremov et al., 2015; Yan et al., 2015; Zalk et al., 2015). The improvements in resolution allowed advances in understanding the structural underpinnings of function, including the location of the binding sites for all four agonists. The sites of Ca^{2+}, ATP, and caffeine binding are close together. The structural changes associated with their actions are consistent with a synergistic effect, whereby binding of Ca^{2+} alone (or ATP and caffeine alone) puts the channel in a "primed" state, which progresses to opening when all agonists (presumably ATP and Ca^{2+} in the physiological situation) are bound (des Georges et al., 2016). Meissner (2017) critically reviewed this evidence.

The central role of CICR in cardiac muscle

The demonstration of CICR in heart muscle was largely done by A. Fabiato and coworkers, who developed methods to exchange solutions rapidly around myocytes with plasma membrane removed. Their series of papers showed that I_{Ca}, the membrane Ca^{2+} current underlying an action potential, is capable of inducing Ca^{2+} release (Fabiato, 1985a); that the CICR response is rapid and sufficient to activate contraction (Fabiato, 1981); that Ca^{2+} release and Ca^{2+} removal occur via separate SR pathways (Fabiato, 1985b); and that Ca^{2+} release is inactivated by Ca^{2+} itself (Fabiato, 1985b). The work provided quantification of the dependencies of both activation and inactivation on $[Ca^{2+}]$ and an initial evaluation of the effects of Mg^{2+}, H^+, and calmodulin (Fabiato, 1983). The results included evidence of activation of Ca^{2+} release by SR Ca^{2+} overload (Fabiato, 1985c, 1992) and advanced the case for a unique Ca^{2+} release pathway with multiple agonists: a single Ca^{2+} release channel. These experiments also pioneered a variety of optical approaches to monitor cytosolic Ca^{2+} transients and other cellular variables putatively associated with Ca^{2+} release (Fabiato and Fabiato, 1979; Fabiato, 1982, 1985d).

This prodigious series of papers used mainly skinned cardiomyocytes. Contemporary work on intact cells and bundles (Cannell et al., 1987; Beuckelmann and Wier, 1988; Niggli and Lederer, 1990a) was largely consistent with Fabiato's conclusions and established that CICR constitutes the sole mechanism of activation of Ca^{2+} release for cardiac EC coupling (Lederer et al., 1989). This consensus is ultimately based on two observations. One is that activation of Ca^{2+} release decays at high V_m, reflecting the decrease in the trigger current I_{Ca} (Näbauer et al., 1989). The other is that, in cardiac muscle, the t- or plasma membrane–SR junctions lack the strict stoichiometry and spatial overlap between Ca_Vs and RyRs thought to be necessary for DICR in skeletal muscle (Block et al., 1988; Tanabe et al., 1990).

The "calcium paradox of control"[4]

The establishment of CICR as chief activation mechanism for Ca^{2+} release in cardiac muscle brought with it a mechanistic problem, first recognized for the heart. Because Ca^{2+} released from the SR adds to the initial trigger of Ca^{2+} entering the cytosol via I_{Ca}, the release response should self-reinforce and reach its maximum in all-or-none fashion. Ca^{2+} release, instead, is graded with depolarization. More dramatically, Ca^{2+} release can be cut short during an action potential by changes in voltage that suppress the trigger I_{Ca}. Michael Stern derived constraints for any model that would successfully reproduce this "paradox of control." In a classic paper, (Stern, 1992a) he first achieved the improbable feat of analyzing by linearization an intrinsically nonlinear system (the cell endowed with CICR). To do it, he used to his advantage the paradox itself—the fact that release is graded, capable of infinitesimal increments in spite of its feedback by CICR—and hence had a linear range of responses. This analysis ruled out the possibility of a common pool system in which trigger and released Ca^{2+} share the same compartment, stressing instead the mechanistic significance of locally inhomogeneous $[Ca^{2+}]_{cyto}$.[5]

Ca^{2+} sparks solve the paradox of control

The interest in local Ca^{2+} gradients generated by the analyses of Stern and the earlier introduction of digital fluorescence Ca^{2+} imaging (Williams et al., 1985; Wier et al., 1987) overlapped in time with the biological application of the first laser-scanning confocal microscopes (Amos et al., 1987). Confocal microscopy was first used in muscle to image immunofluorescence (Somlyo et al., 1988). Confocal Ca^{2+} imaging was possible after the development of high dynamic range fluorescent indicators with fluorescein or rhodamine chromophores (Minta et al., 1989). Confocal imaging of fluo-3 fluorescence was applied simultaneously in 1990 for resolving Ca^{2+} transients in the heart (Niggli and Lederer, 1990b), neurons (Hernández-Cruz et al., 1990), and glia (Cornell-Bell et al., 1990).

[4]The term "calcium paradox" is also used in cardiac physiology to refer to the damage caused by perfusion of isolated hearts with normal saline after exposure to Ca^{2+}-free media (Boink et al., 1976).

[5]Stern's efforts to build a local model of Ca^{2+} release control were not equally successful but still defined concepts (calcium synapse, cluster bomb, and stochastic attrition) that became ingrained in all the mechanistic thinking that ensued.

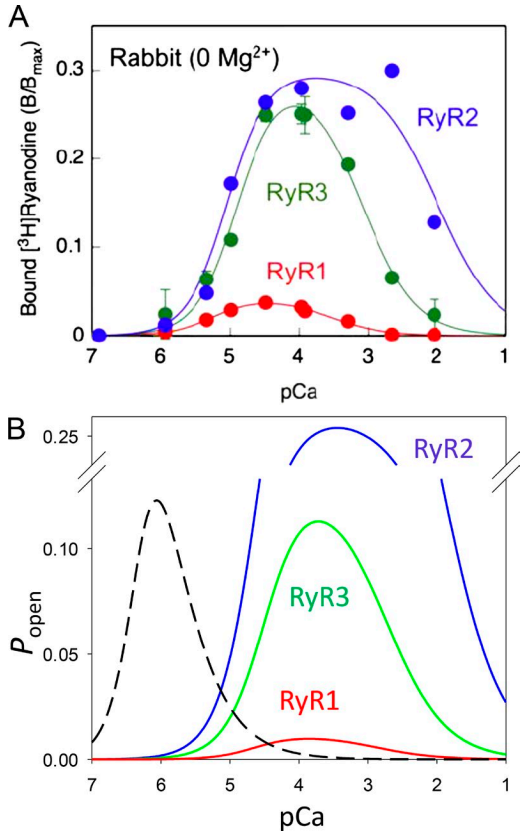

Figure 1. **Ca^{2+} dependence of channel open probability of the three RyR isoforms.** Ca^{2+} dependence measured by ryanodine binding to rabbit muscle preparations. **(A)** Data and fits by Eq. 2 in the absence of extracellular-side Mg^{2+}. **(B)** Best fits in 1 mM Mg^{2+}. Data from Murayama and Kurebayashi (2011); fit parameters listed with their Fig. 2. The dashed curve in B represents the best fit to rate constants of Ca^{2+} release from cardiac SR vesicles, measured with stopped-flow mixing by Sánchez et al. (2003) in the absence of Mg^{2+}. The equation is

$$k = k_{max}\frac{[Ca^{2+}]^{n_a}K_i^{n_i}}{K_a^{n_a}K_i^{n_i} + [Ca^{2+}]^{n_a}K_i^{n_i} + [Ca^{2+}]^{n_i}K_a^{n_a}},$$

with parameter values given in their Fig. 5.

The tired "paradigm-changing" qualifier is still appropriate for the discovery of Ca^{2+} sparks that ensued (Cheng et al., 1993). Like other major advances, this one resulted from the convergence of a strong group of researchers in a field in technical and conceptual movement. It took place in the laboratory of Jon Lederer, who was pioneering the application of confocal microscopy to Ca^{2+} imaging. As recalled by Mark Cannell, a visiting scholar at the time, he and then-student Heping Cheng contributed enhancements to the commercial scanner in use, precise synchronization to stimulus generators, custom-made improvements in signal/noise and sensitivity, original image analysis, and the will to increase laser power with total disregard for cell survival. They found a discrete substructure of Ca^{2+} release in cardiac myocytes, composed by spatially small and temporally brief events immediately dubbed "sparks" (thought of as "sparktifacts" by some, including the editors that rejected the manuscript in *Nature*). These events appeared to be functionally independent (they composed the cellular transient by addition, with individual properties relatively independent of the presence of other sparks). Sparks thus confirmed Stern's conclusion that CICR excludes large common pools and focused mechanistic thinking on the virtues of local pools, or, as they were seductively called, micro- and nanodomains of elevated Ca^{2+}_{cyto} (Llinás et al., 1992; Ríos and Stern, 1997; Fedchyshyn and Wang, 2005).

The discovery of Ca^{2+} sparks "solved" the paradox of control in a general sense. As discussed by Cannell et al. (1995), sparks reconciled the tendency of a CICR mechanism to saturate (it could do so locally) while remaining graded at a cell-wide level. It did not solve the question of mechanism, however, as there was no agreement on even the most basic aspects of Ca^{2+} sparks, including whether they emanate from single or multiple open channels (more on this in the section named Ca^{2+} sparks affirm CICR in skeletal muscle).

CICR in skeletal muscle probed "biophysically"

In 1987, Sidney Fleischer's group clarified the biochemical nature of the Ca^{2+} release channels of the SR (Inui et al., 1987), identifying them both with the ryanodine receptors of heavy SR fractions and the so-called "feet" described by Clara Franzini-Armstrong (1970) in triadic structures. Also in 1987, Gustavo Brum and I identified the receptors of Ca channel blockers present in the t tubules (DHPRs, later named $Ca_V1.1$) as the voltage sensors for EC coupling; meanwhile, the laboratory of Shosaku Numa determined their primary sequence, noting similarities with the voltage-sensitive Na channel, now $Na_V1.4$ (Tanabe et al., 1987). These advances took the focus of research away from the possible roles of CICR, as they strengthened the consensus for a conformational switch (in the DHPR–RyR connection) that translates action potential depolarization to opening of the Ca^{2+} release pathway (Schneider and Chandler, 1973).

But almost at the same time, Franzini-Armstrong's group revealed the peculiar geometric alignment of voltage sensors and Ca^{2+} release channels, which systematically skip every other channel in a checkered double row (Fig. 2; Block et al., 1988). To make functional sense of this arrangement, Ríos and Pizarro (1988) proposed that the RyR channels that lack overlapping voltage sensors (Fig. 2 D, parts labeled C) are operated by CICR.

Although there was no specific functional evidence in favor of this proposal, it also justified kinetic aspects of the waveform of global (i.e., cell averaged) SR Ca^{2+} release flux, derived a few years earlier from the cytosolic Ca^{2+} transients of frog twitch muscle (Baylor et al., 1983; Melzer et al., 1984, 1987). As shown in Fig. 2 D, the waveform elicited by a depolarizing step starts with a peak, followed by a lower, nearly steady plateau. Ríos and Pizarro (1988) proposed that the depolarization first opens the V channels and the ensuing local increase in $[Ca^{2+}]_{cyto}$ causes the C channels to open. Although the activity of C channels was envisioned as short lived and terminated by inactivation (justifying the flux peak), the proposal included that V channels are kept open by the voltage sensors for the duration of the pulse, thus explaining the plateau. This hybrid model was neither formulated quantitatively nor tested experimentally until the following decade (see sections named Ca^{2+} signals of mammalian muscle and The couplons of skeletal and cardiac muscle). Although eventually proven wrong in many respects, the model was a useful stepping stone in the emerging theory.

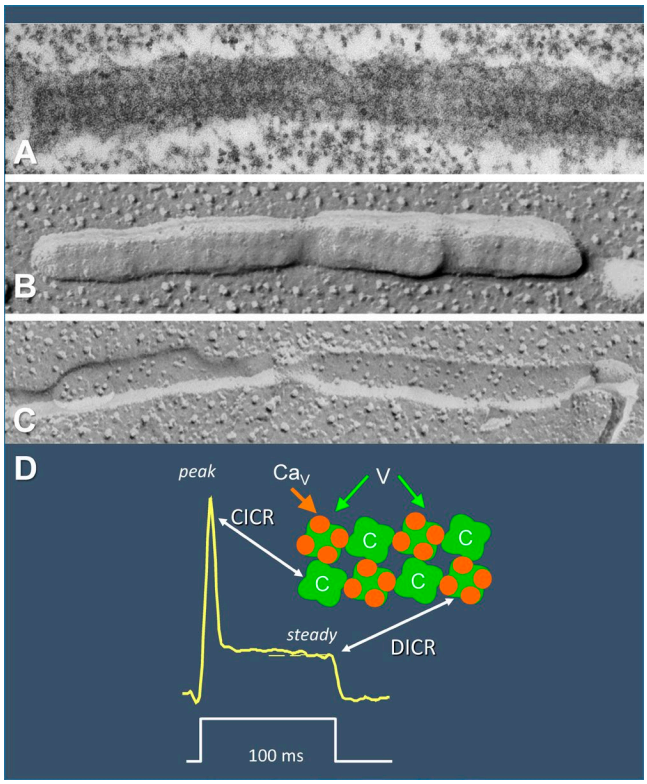

Figure 2. **The relative placement of RyRs and Ca_Vs in muscle. (A)** Thin section showing double rows of "feet" (RyRs). **(B)** Freeze fracture of SR membrane, again showing feet. **(C)** Freeze fracture of t tubule membrane. **(D)** Interpretation by Block et al. (1988) and proposed correspondence between components of release flux and contributions by either V channels (linked to sensors and therefore assumed to engage in DICR) or C channels (assumed to activate by CICR and rapidly undergo CDI). A–C, previously unpublished, are a gift from C. Franzini-Armstrong. D recasts drawings by Ríos and Pizarro (1988).

Starting with Tanabe et al. (1988), Kurt Beam and collaborators confirmed and defined in increasing detail the control of Ca^{2+} release by DHPRs; their approach was the expression of Ca_Vs, wild type or mutated, in primary myotubes derived from pups with a recessive mutation (i.e., dysgenic; Powell and Fambrough, 1973) that causes the deletion of $Ca_V1.1$. This illuminating approach also demonstrated crucial functional differences between the skeletal $Ca_V1.1$ and the cardiac $Ca_V1.2$, which render the latter unable to activate the RyR in the characteristically Ca^{2+}-independent DICR manner, although still capable of activating it via CICR (Tanabe et al., 1990). Combined with EM of the dysgenic myotubes expressing heterologous DNA, the approach demonstrated the ability of $Ca_V1.1$ (but not $Ca_V1.2$) to recreate the junctional tetrads of particles present in the t-tubular membrane of wild-type myofibers (Takekura et al., 1994). Since 1994, the availability of mice engineered for the deletion of RyR1 (Takeshima et al., 1994) was used to demonstrate reciprocal functional effects between RyR1 and $Ca_V1.1$ (Nakai et al., 1996). This feature was evidence that the interaction involves mechanical contact, that the presence of RyR1 is required for the formation of $Ca_V1.1$ tetrads (Takekura et al., 1995), and that these interactions are specific for the skeletal isoforms (RyR1 and $Ca_V1.1$).

These newly understood functional and structural interactions fostered a binary view whereby DICR, a conformationally mediated mechanism, and CICR, a "chemical" transduction, present respectively in skeletal and cardiac muscle and are mutually exclusive. The demonstration of Ca^{2+} sparks in skeletal muscle came to disrupt this consensus.

Ca^{2+} sparks affirm CICR in skeletal muscle

The discovery of Ca^{2+} sparks in cardiac myocytes rapidly led to their description in frog skeletal (Tsugorka et al., 1995) and arterial smooth muscle (Nelson et al., 1995). An essential contribution by Klein et al. (1996) showed that Ca^{2+} sparks of skeletal muscle can be elicited by elevated $[Ca^{2+}]$ in the cytosol, as well as caffeine, a promoter of CICR. Reporting that V_m-evoked events increased their amplitude in a quantized manner (consistent with equal contributions by a variable, small number of channels) as V_m was incremented, Klein et al. (1996) proposed, in agreement with Ríos and Pizarro (1988), that the increase is caused by CICR-mediated recruitment of C by V channels (Fig. 2 D), which grows as more V channels open within sparks.[6]

The number of channels contributing to a Ca^{2+} spark is mechanistically informative; the nearly simultaneous opening of multiple channels requires concerted gating, and Ca^{2+} appears as a likely mediator. The calculation of this number was done by "forward" modeling, which assumes the individual channel flux and calculates how many channels would be needed to build the measured Ca^{2+} spark (Jiang et al., 1999), or by awkwardly named "backward" calculations, which solve the inverse problem of determining the Ca^{2+} flux that caused the measured spark (Ríos et al., 1999). The various calculations led to estimates between 1 and 60 channels. Based on modeling comparisons, Chandler et al. (2003) provided a way of reconciling the wide range of estimates, inferring that sparks involve many more channels in permeabilized myofibers than in intact ones. This conclusion, however, has not been confirmed by direct comparisons on the same setup. Other evidence that multiple channels contribute to a spark include the observation that full sparks are much greater and spatially complex than events induced by imperatoxin, ryanodine, and bastadin 10, toxins that open channels to a known fraction of full conductance (Schneider, 1999; González et al., 2000a). Likewise, large differences were found between sparks and "embers" (i.e., long-lasting events, presumably reflecting the opening of single channels, which may precede or follow sparks; see additional discussion below; González et al., 2000b).

The distribution of measured spark amplitudes was also scrutinized to unveil mechanisms. The apparent quantization and presence of local maxima (modes) in this distribution (Klein et al., 1996; Shirokova and Ríos, 1997) were first naively interpreted as reflecting a stereotypical activation of multiple channels (which implies CICR). This interpretation requires

[6]Klein et al. (1996) also provided two simple technical insights that helped "sparkologists": the use of a confocal aperture (pinhole size) >1 Airy disk (theoretically ideal for resolution) to maximize sensitivity and the advantage of scanning planes close to the cell surface to minimize scattering. The study of $[Mg^{2+}]$ dependence by Schneider's group (Lacampagne et al., 1998) additionally defined conditions for the collection of large numbers of sparks. In a later paper, Klein et al. (1997) amended their initial conclusion, demonstrating that the frequency but not the size of sparks increased with depolarization.

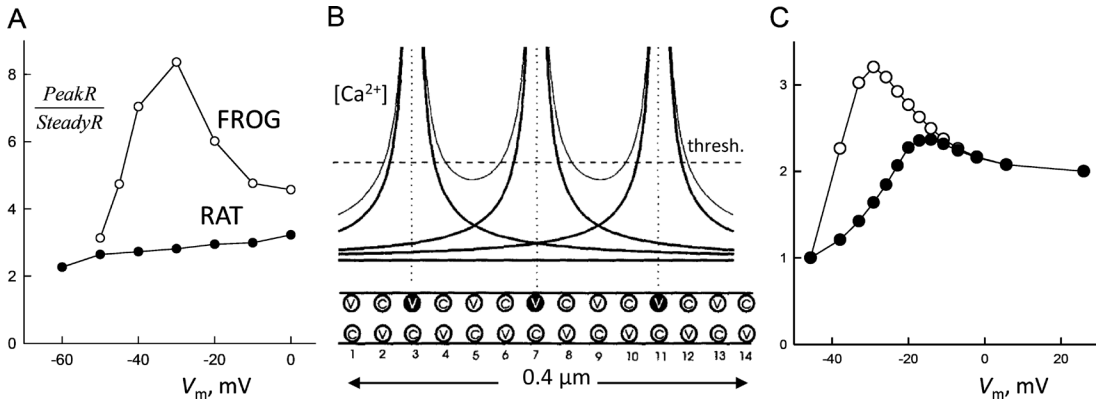

Figure 3. **Voltage dependence of the ratio of release flux measures.** Flux measures *P* (peak) and *S* (steady) are defined in Fig. 2. **(A)** Measured ratios *P*/*S* in frog and rat muscle. **(B)** A model in which the peak component is attributed to flux through C channels activated by Ca^{2+} domains near open V channels. The graphs illustrate components provided by three open V channels (thick lines) and their sum (thin lines). C channels open when $[Ca^{2+}]$ goes above a threshold level (dashed line). **(C)** The model accounted qualitatively for the modal dependence *P*/*S* (V_m) in frog muscle (open circles). A simple change in parameters that made the C channels less excitable did not fully account for the qualitative characteristics of the dependence in the rat (filled circles). Details in Shirokova et al. (1996).

that the distribution of Ca^{2+} sparks remains quantized after imaging by the microscope. This condition does not hold in general; four papers advanced the theory of confocal image acquisition later in the decade, proving that the observed (imaged) distributions cannot retain modes present in the actual (true) distributions. Pratusevich and Balke (1996) predicted that modes would disappear upon confocal sampling. Izu et al. (1998) and Cheng et al. (1999) independently showed that sparks of fixed amplitude α, when occurring at random distances from the focal plane of a confocal imaging system, will give rise to an observed distribution of amplitudes *a* of density $f(a) = k/a$ (where *k* is constant). This relationship, valid for $a \le \alpha$, implies that the apparent amplitude distribution will be monotonically decaying, regardless of the true amplitudes, single or multiple, of the imaged events. Ríos et al. (2001) generalized the study to find that (under reasonable assumptions) the observed distribution $f(a)$ and the true distribution $g(a)$ are related by

$$\sigma^2(a)\,g(a) = k\frac{d(af(a))}{da}, \qquad (3)$$

where *k* is a constant.

This theory allowed the back calculation of $g(a)$ starting from the observed $f(a)$. Thus corrected, the amplitude distribution had a mode in most cases (González et al., 2000a). In the same fibers, much smaller events could be elicited, which in cardiac muscle were dubbed "quarks" (Lipp and Niggli, 1996) and in skeletal muscle appeared as embers after sparks evoked by agonist drugs (González et al., 2000a).

The demonstration of modes in the distribution of amplitudes, together with the presence of much smaller embers and quarks, confirmed that sparks are caused by the opening of multiple channels. Moreover, modes in $g(a)$ were accompanied by modes in the distribution of rise times. The implications are profound; as discussed by Bridge et al. (1999), Cannell and Soeller (1999), and González et al. (2000b), modes in rise times rule out Markovian channels gating reversibly, i.e., with rate constants depending on the present state only and satisfying microscopic reversibility (Colquhoun and Hawkes, 1995). Later work demonstrated that neither cardiac Ca^{2+} sparks (Wang et al., 2002) nor spontaneous events of Ca^{2+} release in frog skeletal muscle (Rengifo et al., 2002) could be caused by channels gating reversibly. The conclusion from these studies was that sparks result from multiple channels gating concertedly, which requires a synchronization mechanism, and irreversibly, which requires an energy source. CICR appeared as the obvious concerting mechanism; the source of energy is presumably the SR-to-cytosol Ca^{2+} gradient, which couples to the gates via CICR.

In spite of the consensus that Ca^{2+} sparks are a manifestation of CICR, whether sparks (and CICR) were relevant to skeletal EC coupling remained in dispute. Examination of the global, cell-wide Ca^{2+} transients provided some answers.

Ca^{2+} signals of mammalian muscle

The early studies of Ca^{2+} sparks overlapped in time with the extension to mammals (Delbono and Stefani, 1993; Garcia and Schneider, 1993) of techniques developed on frog muscle to determine flux of Ca^{2+} release. Using these techniques, Shirokova et al. (1996) found a striking difference between muscles of amphibians and rodents. As shown in Fig. 3 A, in the frog, the dependence of the peak amplitude of Ca^{2+} flux (*P*) with applied voltage V_m differs sharply from that of the steady level (*S*) reached after the peak. Consequently, the ratio *P*/*S* rises as V_m increases, reaches a maximum at about −40 mV, and decays at higher V_m. In contrast, $P(V_m)$ is nearly proportional to $S(V_m)$ in the rodents, so that *P*/*S* only rises slowly throughout the voltage range. The simple calculation of Fig. 3 (B and C), a hybrid of DICR and CICR whereby the local Ca^{2+} domains generated around open V channels add up to activate intercalated C channels, explained without added assumptions the peculiar dependence of *P*/*S* on V_m observed in frogs.

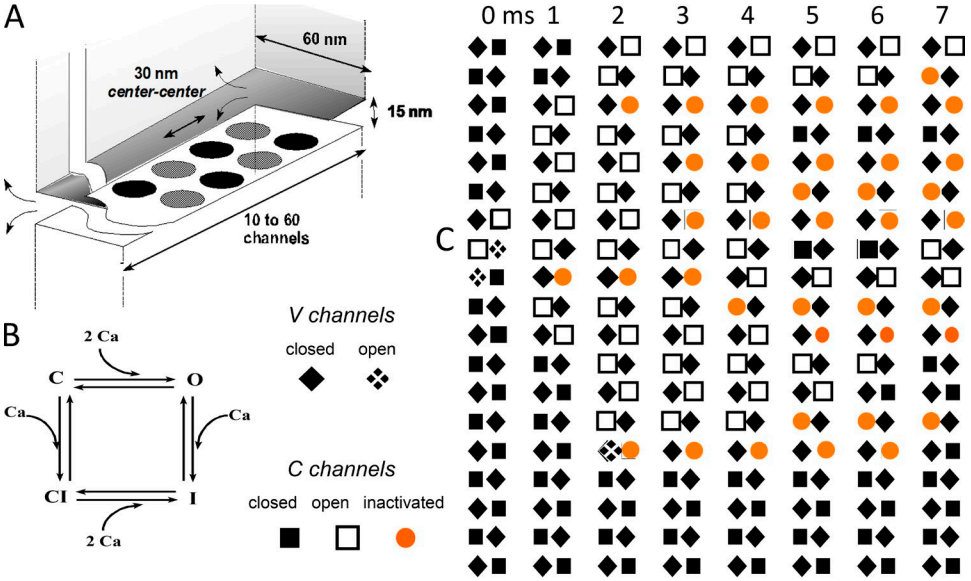

Figure 4. **Self-consistent simulation of a hybrid DICR–CICR model of Ca^{2+} release. (A)** Geometry. Ca^{2+} is released via alternating V and C RyRs in double rows. Local $[Ca^{2+}]$ is determined by Ca^{2+} diffusing inside a junctional gap and surrounding wide cytosol. **(B)** A diagram depicts the model for activation and inactivation of C channels. V channels depend on V_m according to a quantitative allosteric scheme (Ríos et al., 1993). They are assumed not to inactivate. **(C)** Successive snapshots of the array of channels in one Monte Carlo realization. An event started at 0 ms with voltage activation of two channels progresses via CICR along the array. The Ca^{2+} transient associated with this event has spatial and temporal properties of a Ca^{2+} spark. Note that it was sufficient with the inactivation of one channel ahead of the activation wave to stop its downward progression. From Stern et al. (1997).

The calculation of Shirokova et al. (1996) included drastic simplifications, as it assumed static concentration profiles and ignored any contribution of C channels to the local $[Ca^{2+}]_{cyto}$. It reproduced, however, the essential nonlinearity of the system at low V_m (as arising from an additive interaction of concentrations facing a binary activation threshold). It also explained the decay of P/S at higher V_m, as the CICR contribution stops increasing when all C channels are open.

The observations revealed a major difference between taxa. The flat P/S (V_m) of rodent muscle, which according to the model reflects an absence of CICR, together with evidence of a greater density of RyRs (relative to Ca_Vs) in amphibians (Bers and Stiffel, 1993; Margreth et al., 1993; Anderson et al., 1994), suggested that the amphibians' CICR is carried via their excess RyRs. This proposal matched emerging evidence of the presence of two RyR isoforms in amphibian (Lai et al., 1992) and avian muscle (Airey et al., 1990). These isoforms, named α and β, are respectively orthologues of mammalian skeletal RyR1 and of RyR3, which is expressed in neurons and other cells but not in most skeletal muscles (Conti et al., 1996; Ottini et al., 1996). In subcellular preparations, α and β activated respectively by DICR and CICR (Ivanenko et al., 1995; Kashiyama et al., 2010). The obvious expectation was that frogs' CICR be carried by their β isoform.

Shirokova et al. (1996) also reported that peak release flux is greater in frogs than in mammals under comparable conditions. This difference is consistent with the excess channels and the putative presence of CICR in amphibians. At a Biophysical Society meeting, Elizabeth Stephenson stood by Shirokova's poster and suggested a compelling teleological explanation of these differences. She noted that although frog muscles have one triad per sarcomere, located near the Z disk, mammals have two, placed much closer to the target for the released Ca^{2+} (the region of overlap between thick and thin filaments). Given the nonlinear relationship between distance and time in diffusion processes, the dual triads of mammals should allow for a tighter control of contraction, with less released Ca^{2+} and no need for the intrinsically explosive CICR mechanism.

The couplons of skeletal and cardiac muscle

The inconsistencies in the hybrid DICR/CICR calculation by Shirokova et al. (1996) mentioned in the previous section were removed by Stern et al. (1997), who computed all channel interactions dynamically using the Monte Carlo simulation and the more appropriate geometry shown in Fig. 4. The simulations derived global Ca^{2+} flux by averaging the stochastic release events produced by sets of RyRs and their controlling Ca_Vs. The number of RyRs in these sets was chosen to match the size of t–SR junctions. Ca_Vs activated alternate RyRs (V type) according to allosteric interaction rules established earlier by comparing release activation and voltage sensor charge movements (Ríos et al., 1993). The Monte Carlo runs produced realizations, Ca^{2+} release events leading to local Ca^{2+} transients. Fig. 4 includes a typical realization in which activation, started at two V channels, propagates by CICR along the set of RyRs.

The local events produced were thus passable copies of experimental sparks. Their averages reproduced features of the cell-wide records, including the observed mode in P/S (V_m). The success of the model endorsed the coexistence of DICR and CICR and Stern's conclusion that control is local.

The simulations also showed that, under reasonable parameter values, it is sufficient with inactivation of one C channel to interrupt propagation (in Fig. 4, a single inactivated channel prevented propagation to the lower third of the set). This fragility implies that activation in one side of a triad can hardly propagate to the other side. The release channels and voltage sensors on one

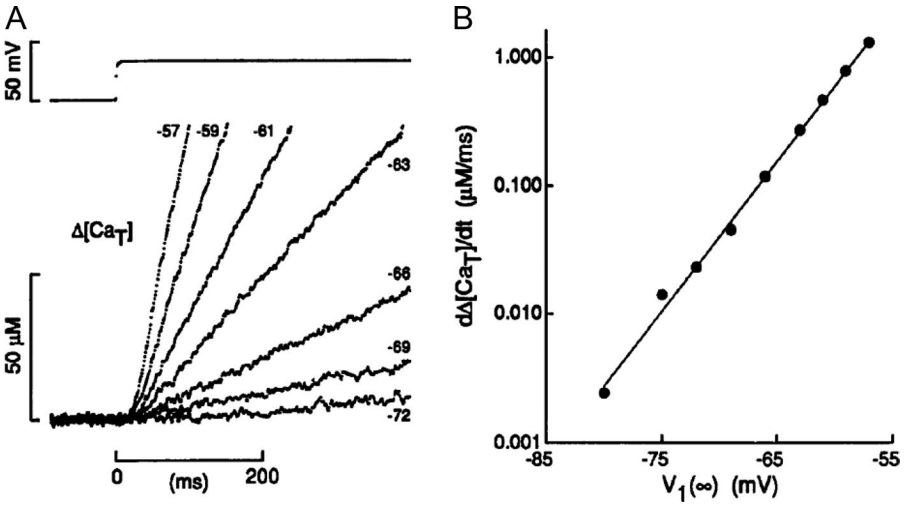

Figure 5. **Ca^{2+} release near the resting potential. (A)** Evolution of total released calcium [Ca_T] upon application of pulses (top) at V_m near the resting potential. **(B)** Log of the slope of plots in A versus applied voltage. The slope $(3.7\ mV)^{-1}$ corresponds to an effective sensing charge of 6.7 *e*. At V_m greater than −55 mV, the slope was reported to diminish. From Pape et al. (1995).

side of a triad thus behave as a functional unit, which was named "couplon." The definition was immediately extended to cardiac muscle (Stern et al., 1999), where the interaction between Ca_Vs and RyRs was modeled as having been purely caused by CICR, although the RyR–RyR interaction could be mediated by both CICR and conformational signaling.

That RyR2 channels may interact allosterically was contemplated often in later work but never supported by hard evidence. Recently, studies of cardiac couplons showing that the clusters of RyRs have fewer elements, with numbers and geometric arrangement more variable than originally thought (Baddeley et al., 2009; Soeller and Baddeley, 2013; Asghari et al., 2014), have made conformational RyR2–RyR2 interaction less attractive. However, the cryo-EM studies of Cabra et al. (2016) have showed that one of the configurations in which RyR2s mutually interact in vitro involve an apparent overlap, consistent with an intricate contact that might support allosteric interactions if present in vivo.

Additionally, progress in biochemical and imaging studies demonstrated the presence of many smaller molecules that bind to RyRs and Ca_Vs. Initially, the attention was placed on the SR membrane protein triadin (Kim et al., 1990) and the luminal protein calsequestrin (MacLennan and Wong, 1971); a long and growing number of proteins are now known to interact with Ca_Vs, RyRs, or both. The couplon definition was accordingly generalized to include them (Ríos et al., 2015). Whether and how altering any of these proteins modifies the couplon function are the subject of active research (Rebbeck et al., 2014). This line of work was punctuated recently by the demonstration of an essential role of the adapter protein Stac3 in the activation of RyR1 by voltage (Horstick et al., 2013; Nelson et al., 2013; Polster et al., 2016) and the enormous enhancement that it produces in the expression of heterologous $Ca_V1.1$ in *Xenopus laevis* oocytes (Wu et al., 2018).

Direct tests of a physiological role of CICR in skeletal muscle

The evidence for CICR operation in skeletal muscle reviewed so far is largely indirect. The many experiments done to more directly test and quantify the putative physiological role of CICR will be reviewed in two groups: those involving the introduction of a Ca^{2+} buffer inside cells, realized largely in the 1980s and 1990s, and those using molecular manipulations, developed after the year 2000.

Probing CICR with Ca^{2+} buffers

The first use of a cation buffer to probe muscle contraction was communicated by Emil Bozler in this journal, in 1954. Bozler used EDTA to remove Ca^{2+} from membrane-permeabilized muscle. The more selective buffer EGTA (Weber and Winicur, 1961) later allowed for setting [Mg^{2+}] higher to probe the roles of Ca^{2+} and Mg^{2+} in controlling the interactions of actin and myosin (Herz et al., 1969). EGTA was first applied intracellularly by Portzehl et al. (1964), who used the contractile response to solutions of different [Ca^{2+}] injected into muscle cells to establish that resting $[Ca^{2+}]_{cyto}$ is <200 nM. EGTA was again used as monitor, this time of released Ca^{2+}, in a method called EGTA–phenol red (Pape et al., 1995) based on the near stoichiometric displacement of H^+ by Ca^{2+} as it binds to EGTA. That deprotonation of EGTA must precede or accompany Ca^{2+} binding both slows the reaction kinetics and makes it pH dependent; these inconveniences led to the development of BAPTA (Tsien, 1980) and its use (Marty and Neher, 1985) as a much faster Ca^{2+} chelator.

Conversely, Ca^{2+} monitors proved useful as buffers. In an early application, Kovacs et al. (1983) calculated the cytosolic concentration of endogenous Ca^{2+} buffers from the changes in the decay kinetics of Ca^{2+} transients induced by known concentrations of the absorption indicator Antipyrylazo III; their work also called attention to the inescapable perturbation of Ca^{2+} transients by its monitors.

Baylor and Hollingworth (1988) pioneered the use of the buffer properties of monitors to probe mechanism. Large quantities of Fura-2 in frog myofibers changed the dye signals in ways consistent with an increase in action potential–induced Ca^{2+} release, seen as resulting from a reduction of the inactivation of Ca^{2+} release by Ca^{2+} (Ca^{2+}-dependent inactivation [CDI]). This affirmation of CDI indirectly negates a major role of CICR. Also weighing against this role was the reported absence of effects of the specific CICR inhibitors procaine and adenine (Endo, 1985) and the anemic rates of Ca^{2+} release evoked by Ca^{2+} either in skinned frog fibers (Murayama et al., 2000) or in vesicular SR fractions of rabbit muscle (Meissner et al., 1986).

Additional arguments against the physiological operation of CICR came from the laboratory of W.K. Chandler. Pape et al. (1995) used the EGTA–phenol red technique to accurately define the V_m dependence of release flux at V_m near the resting potential. Fig. 5 shows superimposed changes in total cytosolic Ca^{2+} concentration upon long-lasting depolarization at the V_m values listed. In B, the rate of change of $[Ca_T]$ is shown to vary exponentially with V_m, a dependence consistent with activation by voltage, exclusive of other mechanisms (Almers, 1978). In experimental observations (Klein et al., 1996; Lipp and Niggli, 1996), CICR manifests as a nonlinear additive component to DICR. The observed exponential dependence is therefore indicative of exclusive control of release by a voltage sensor in the negative range of voltages tested. Pape et al. (2002) would later show how to reconcile these results with the operation of CICR (also see Fig. 7).

Calcium concentration near arrays of open channels

The articles by Pape et al. (1995, 1998) constituted milestones in the analytical description of $[Ca^{2+}]_{cyto}$ near open Ca^{2+} channels. In earlier attempts, Neher (1986) and Stern (1992b) derived an expression for the steady increase in concentration $\Delta [Ca]_s$ versus distance r from the mouth of a point source (channel) of flux ϕ in an isotropic medium containing a buffer at high concentration (say, 20 mM EGTA):

$$\Delta [Ca]_s = \frac{\phi}{4\pi D r} e^{-r/\lambda}. \quad (4)$$

In this expression, the first factor represents the distribution in the absence of buffer. The effect of the buffer is to "smear" the distribution by an exponential of space constant λ, determined by the ion's diffusion constant, D, and its rate of binding to the buffer (which is the inverse of the mean time required by the buffer to complex Ca^{2+}: $k[EGTA] \equiv \tau^{-1}$).

$$\lambda = \sqrt{D/k[EGTA]}. \quad (5)$$

The approach used by Stern (1992b) removed the high buffer simplification (and showed that it was good in most cases of interest). Pape et al. (1995) then found an expression for the concentration profile at times t after channel opening, which turned out to be equal to the steady-state profile (Eq. 4) multiplied by a function F of time and space that starts at value 0 at $t = 0$ and tends to 1 as time increases:

$$F(r,t) = 0.5\left\{\mathrm{erfc}\left(\frac{r}{\sqrt{4Dt}} - e^{-\sqrt{t/\tau}}\right) + e^{2r/\lambda}\mathrm{erfc}\left(\frac{r}{\sqrt{4Dt}} + e^{-\sqrt{t/\tau}}\right)\right\}. \quad (6)$$

Under the buffering conditions of interest and at distances of <0.5 µm, F is very close to 1 (and the steady solutions are therefore appropriate).

One Ca^{2+} flux waveform leads to two opposite conclusions

Using the tools above, Pape et al. (1998) described analytically $[Ca^{2+}](r)$ near a set of active Ca^{2+} release channels with couplon geometry, immersed in a medium with realistic buffers. They

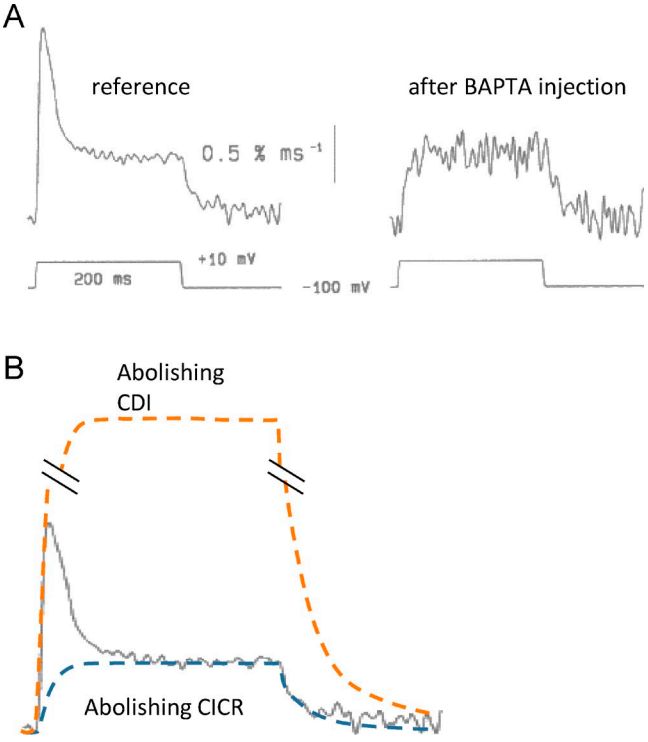

Figure 6. **The effect of a fast Ca^{2+} buffer on calculated Ca^{2+} release flux. (A)** Flux calculated by Jacquemond et al. (1991) from cytosolic Ca^{2+} transients measured in frog myofibers before and after injection of BAPTA. **(B)** Reference record from A with superimposed lines depicting predictions of the records that would result if BAPTA abolished inactivation (orange) or activation by Ca^{2+} (blue). The calculations of flux by Jacquemond et al. (1991) are consistent with abolition of CICR.

compared the effect of the presence of buffers, which reduce $\Delta[Ca^{2+}]_{cyto}$ more effectively at greater distances from the open channel, with that of SR depletion, which affects the unitary Ca^{2+} current and therefore reduces $\Delta[Ca^{2+}]_{cyto}$ evenly at all distances. As total SR Ca content ($[Ca]_{T,SR}$) was reduced, the fraction of total SR Ca released by an action potential increased greatly, suggesting reduction of CDI. With one exception,[7] the experiments failed to reveal the effects predicted if CICR was operative. Application of Eqs. 5 and 6 located the inhibitory site at <22 nm from the open channel; that is, on the same RyR or the nearest neighbor in the couplon.

Surprisingly, M.F. Schneider and colleagues reached the opposite conclusion from a similar approach. Injection in frog myofibers of BAPTA, together with Fura-2 (which served as monitor of both the injection of BAPTA and the change in cytosolic Ca^{2+}), altered calculated Ca^{2+} release flux in ways consistent with inhibition of CICR (Jacquemond et al., 1991; Csernoch et al., 1993). After injection of the buffers, the waveform of Ca^{2+} release flux lost its peak (Fig. 6 A). As illustrated in Fig. 6 B, two interpretations of the blunting effect were possible: in the hypothesis of a hybrid activation mechanism, the loss of the peak could simply reflect loss of the CICR component; alternatively, if the buffers just prevent CDI, a loss of peak will also ensue, caused by loss of the decay that follows the peak.

The disparate interpretations of the loss or blunting of flux peak emerged from different methods to evaluate the flux

[7]A single item of evidence in favor of CICR emerged in these experiments: Ca^{2+} release flux decreased by about 50% upon increasing the concentration of Fura-2 in the cytosol from 5–6 to 7–8 mM (Jong et al., 1993). This decrease, however, could just reflect a deleterious effect of the dye.

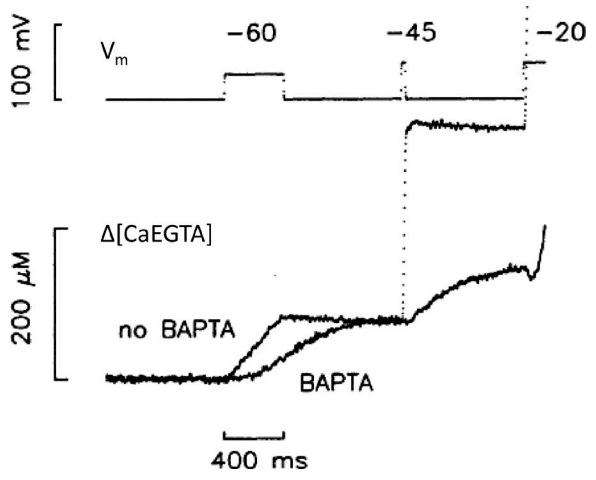

Figure 7. **The effect of BAPTA on V_m-elicited flux is strongly dependent on the applied voltage.** Δ[CaEGTA] measures total Ca released. The difference in amount at −60 mV is nil (BAPTA delays the transfer of released Ca^{2+} to EGTA). It is maximal at intermediate voltages (−45 mV in this case). This evidence of V_m dependence of a putative CICR component is consistent with the modal V_m dependence of *P/S* illustrated with Fig. 3. From Pape et al. (2002).

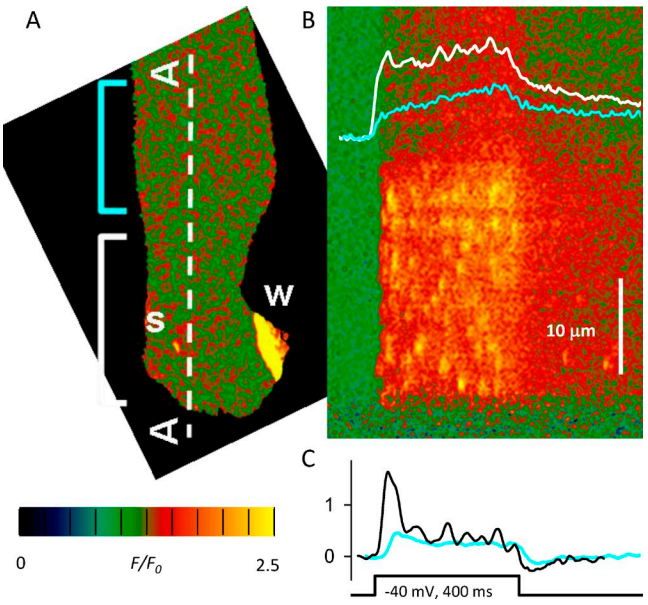

Figure 8. **Expression of RyR3 in a myofiber from adult mouse. (A)** Isolated myofiber held at resting potential under patch clamp. Fluo-4 reveals abortive Ca waves originating at a swollen nucleus (W). Simultaneously, spontaneous sparks (s) appear randomly, exclusively in the fiber segment within the white bracket. **(B)** Confocal scan along line A–A in A. An applied pulse of −40 mV elicits a response that includes sparks in the segment within the white bracket, but is devoid of sparks in the adjacent segment (cyan bracket). The Ca^{2+} transient is greater in the segment with sparks (white trace). **(C)** The calculated Ca^{2+} release flux includes a peak in the sparking region (black trace) that is not present in the sparkless area (cyan). Modified from Pouvreau et al. (2007).

magnitude, which resulted in strikingly different scalings of a similar waveform (Fig. 6 B, blue and red traces). Although the disagreement was never truly solved, Pizarro and Ríos (2004) tried to reconcile the conclusions by applying both methods (the removal analysis of Melzer et al., 1987, used in the Schneider laboratory, and the EGTA–phenol red method of Chandler and colleagues) simultaneously to the same frog cells. Their conclusion essentially split the difference, finding buffer-induced reductions in both CICR and CDI.

In a further stab at reconciliation, Pape et al. (2002) recorded Ca^{2+} flux in frog myofibers under voltage clamp while curtailing the increase in $[Ca^{2+}]_{cyto}$ near open channels, either by BAPTA in the cytosol or depleting $[Ca]_{T,SR}$. Their novel idea was that this change should reduce the putative CICR contribution in a V_m-dependent manner, much in the same way as *P/S* depends on V_m and for the reasons proposed in Fig. 3. The result (Fig. 7) was that BAPTA, entering the cytosol from cut fiber ends, markedly reduced the Ca^{2+} released at intermediate V_m, although at −60 mV the amount remained the same (the kinetic difference shown is expected, as Ca^{2+} must transit to EGTA from the faster reacting BAPTA). The selective effect at −45 mV is consistent with both the V_m dependence of *P/S* (Fig. 3 A) and the results in Fig. 5, suggesting that release at −60 mV reflects DICR and, at higher V_m, recruits an extra CICR component.

On balance, the application of intracellular buffers suggested but did not demonstrate a significant contribution of CICR to Ca^{2+} transients for EC coupling in skeletal muscle. Although Ca^{2+} sparks were believed to involve CICR, fully establishing their mechanism and their contribution to physiological signals required other approaches.

[8]An implicit assumption was that feet are homotetramers (i.e., that isoforms 1 and 3 do not combine in tetramers). This guess was upheld by the experiments of Xiao et al. (2002), which established that RyR2 can instead combine with RyR1 and RyR3.

Probing CICR with heterologous expression

As described earlier in this article, this approach started with the expression of $Ca_V1.1$ and RyRs in primary myotubes. Consequently, it was not useful at first to establish the physiological role of CICR in adult muscle. Two advances at the turn of the century changed this situation. First, Felder and Franzini-Armstrong (2002) noted parajunctional feet (PJFs; RyRs located outside the t–SR junctions). PJFs were found in muscles containing both isoforms 1 and 3 (including frog fast-twitch and fish swim muscles), and in RyR1-only muscles, feet appeared exclusively in a double row at the junction (junctional feet [JFs]), in interaction with Ca_Vs. Felder and Franzini-Armstrong proposed that PJFs are of isoform 3. Based on the different clustering patterns of JFs and PJFs, they also surmised that JFs are exclusively of isoform 1.[8]

This evidence forced revision of the models of Shirokova et al. (1996) and Stern et al. (1997) because the assumed double row geometry was wrong for the (frog) cells that originate the simulated phenomena. Instead, the geometry was adequate for fast-twitch mammalian muscle, which has no PJFs. The simulation of events was wrong there as well, as no sparks were observed in mammalian muscle under stimuli that would cause them in the frog (Shirokova et al., 1998; Csernoch et al., 2004).

Evidence of different properties of RyR1 and RyR3 was gathered in myotubes from RyR (1 or 3)-null mouse embryos (Conklin et al., 2000) and by expressing either isoform (Ward et al., 2000, 2001) in a dyspedic myogenic cell line devoid of RyRs (Moore et al., 1998). Although both isoforms generated spontaneous

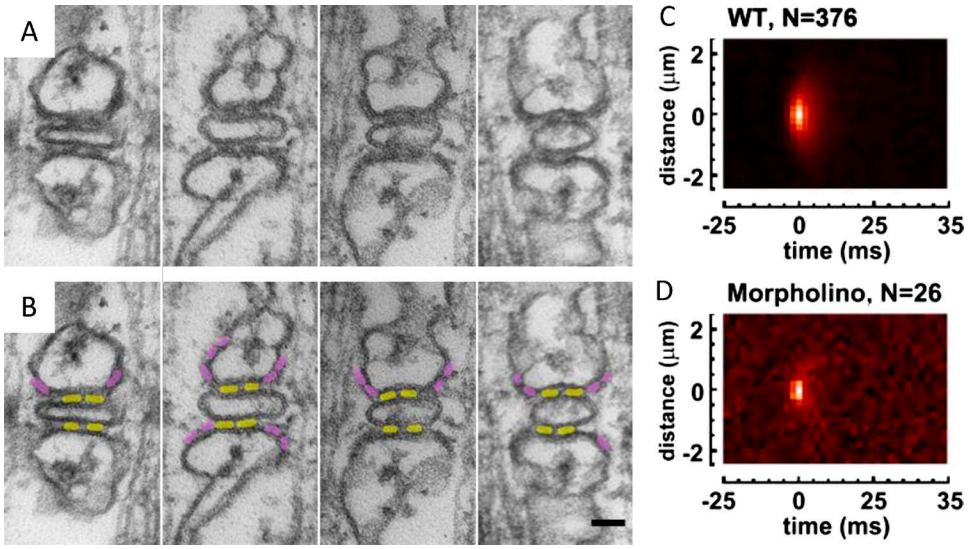

Figure 9. **Junctional and parajunctional feet in muscle of young zebrafish embryo.** Triads shown in transversal section. **(A)** EM images. **(B)** Interpretive colorization. Parajunctional feet, marked purple, disappear upon injection of morpholinos (not depicted in this figure). **(C)** Average of reference sparks. **(D)** Average of events in morphant larvae, which are scarce and smaller. Modified from Perni et al. (2015). Bar, 50 nM.

spark-like events, RyR3 did it better, but failed to activate by membrane depolarization.

After these advances and using a technique to express heterologous proteins in muscles of adult mice optimized by DiFranco et al. (2006), two groups endeavored to express RyR3 in adult murine muscles, with striking results. Pouvreau et al. (2007) found that the expression caused sparks to appear, both spontaneously and under depolarization, in myofibers initially devoid of events. As shown in Fig. 8 A, the V_m-elicited sparks appeared in segments of the myofiber located near perinuclear regions actively synthesizing the foreign protein, where spontaneous events were frequent. This segmental expression permitted a comparison with nearby segments that were silent, i.e., with no evidence of exogenous activity. The sparks activated by voltage (Fig. 8 B) contributed to a peak of Ca^{2+} release (Fig. 8 C) much greater than that present in the silent regions. Using the same approach, Legrand et al. (2008) similarly found segmental expression, spontaneous activity confined to areas of high RyR3 expression, and a more prominent peak of Ca^{2+} release, but did not detect V_m activation of sparks. This difference notwithstanding, the experiments established RyR3 as a source of Ca^{2+} release not directly activated by voltage. Additionally, the RyR3-dependent modification of the global V_m-induced Ca^{2+} flux waveform and the observation of V_m-stimulated spark-like events completed the demonstration of a hybrid DICR–CICR mechanism with separate RyR1 and RyR3 components.

In spite of these advances, doubts remained regarding the nature of the Ca^{2+} sparks and their contribution to physiological Ca^{2+} release. Two groups set out to address these questions directly. Our laboratory took advantage of a novel dual confocal scanner (Zeiss), which scans simultaneously with two separately focused lasers. We used one scanner to deliver a local Ca^{2+} bolus at selectable locations via photorelease of caged Ca^{2+} while monitoring the response with the other (Figueroa et al., 2012). Two-photon excitation of a novel high efficiency "cage" resulted in a bolus (named SLIC, for synthetic local increase in calcium) that was adjustable to mimic the spatial size and duration of a Ca^{2+} spark. The SLIC was delivered outside the fibers (at 2 μm from the plasma membrane, permeabilized by saponin) both to avoid direct stimulation by the uncaging irradiation and to allow quantitative measurement of stimulus $[Ca^{2+}]$ separate from the response. With frog muscle, SLICs consistently stimulated Ca^{2+} release. The stimulus $[Ca^{2+}]$ level measured at the cell boundary could be as low as 180 nM and still elicit a response. In contrast, mouse myofibers did not produce measurable release even with the most intense SLICs (8 μM at contact point) and with the inhibitory Mg^{2+} set at unphysiologically low concentrations. In view of this low threshold $[Ca^{2+}]$, we concluded that CICR occurs during normal EC coupling in frog muscle and does not work in mammals under physiological conditions.

The question was also addressed directly by EM imaging and functional probing of zebrafish "morphant" larvae, which were injected at the one-cell stage with a morpholino that impedes expression of RyR3 (Perni et al., 2015). Wild-type muscle showed normal JF and PJF arrays (Fig. 9, A and B) as well as frog muscle–like Ca^{2+} sparks. Morphant muscle instead had an 80-fold lower frequency of sparks, which were smaller (Fig. 9, C and D). Most importantly, PJF were nearly absent in morphants. These observations establish that RyR3 are parajunctional and generate sparks by CICR. As to the physiological relevance of CICR, the work was inconclusive because it did not find deficits in the swimming behavior of morphant larvae.

The failure of RyR1 to engage in CICR is surprising, as in bilayers RyR1 and RyR3 open at about the same $[Ca^{2+}]$ (Fig. 1; also see section named The RyR in subcellular preparations). This discrepancy has been addressed; working with vesicular fractions, Murayama and Ogawa (2001) found that the binding of ryanodine, as a measure of channel activation, was 20 times greater for the β than the α isoform (Fig. 1). This result suggests that the ability for CICR of α/RyR1 is inhibited in the SR vesicles by interactions lost in the process of molecular isolation.[9]

Shirokova et al. (1999) reported a striking segregation of Ca^{2+} events in primary mouse myotubes. In response to depolarization, myotubes produced Ca^{2+} release devoid of sparks,

[9]In the formalism of Eqs. 1 and 2, the interactions would reduce $F_{a,max}$ while sparing f_i.

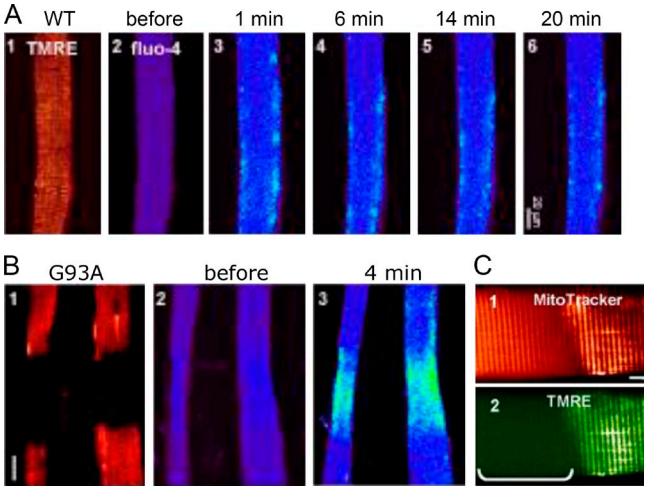

Figure 10. **CICR in amyotrophic lateral sclerosis. (A)** Single myofiber from a reference mouse. **(B)** Myofibers from a mouse constitutively expressing an amyotrophic lateral sclerosis–linked SOD1 mutation. Absence of tetramethyl rhodamine ethyl ester (TMRE) fluorescence marks segments lacking mitochondrial transmembrane potential. Cytosolic Fluo-4 reveals ECRE in response to osmotic stress. In the mutant, ECRE evolve to global Ca^{2+} release, which stops at the edge of the damaged segment. **(C)** MitoTracker staining indicates that mitochondria are present in the damaged regions. Eventually, the lesions progress to mitochondrial destruction. Modified from Zhou et al. (2010).

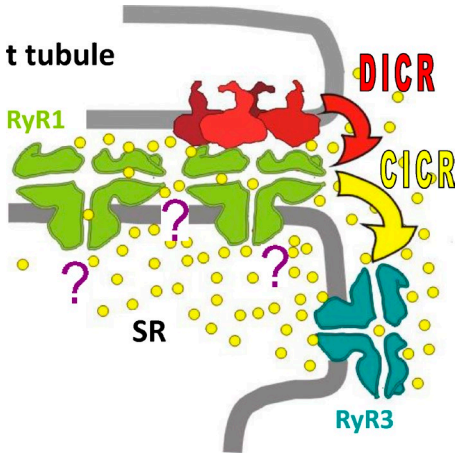

Figure 11. **Consensus and questions. RyR1 or α, located exclusively at t–SR junctions, are activated conformationally by $Ca_V1.1$ (DICR).** RyR3 or β, absent in most mammalian muscles, are located parajunctionally and activated by Ca^{2+} (CICR). Question marks represent the unknown role of uncoupled junctional RyR1 (C channels), the unconfirmed possibility of allosteric RyR–RyR interactions, and the evolving quest on RyR control from within the SR. Modified from Pouvreau et al. (2007).

typical of the adult. Sparks only occurred in areas unresponsive to depolarization. This segregation occurred in both wild-type and RyR3-null myotubes, hence proving that RyR1 are capable of both Ca^{2+} sparks and sparkless function. Consistent with this observation, Chun et al. (2003) reported that both RyR1 and 3 can generate sparks in embryonic and early postnatal myofibers. Later, it was found that sparks occur only where t tubules are absent (Zhou et al., 2006; Brown et al., 2007). The repression of spontaneous events at t tubules was associated with the presence of $Ca_V1.1$, as it did not occur in cells cultured from $Ca_V1.1$-less muscular-dysgenic (*mdg*) mice (Zhou et al., 2006) or in regions with disorganized t tubules of adult myofibers undergoing dedifferentiation in culture (Brown et al., 2007). These observations confirmed that the intrinsic sensitivity of RyR1 for activation by Ca^{2+} is prevented when the channels join in the DICR-capable couplon.

CICR in disease

Although CICR does not operate in most mammalian muscles under physiological conditions, its operation under altered or diseased situations is now evident. Kirsch et al. (2001) described conditions to systematically observe local events in muscle of mice and rats. These were dubbed elementary Ca^{2+} release events (ECRE) because, unlike sparks, they appeared in a variety of spatial shapes and time courses. Because large ECRE require Ca^{2+} release through multiple channels, they should, in principle, involve CICR. The conditions for their production included mechanically removing or chemically permeabilizing the plasma membrane, an observation consistent with the inhibition by Ca_Vs proposed earlier to explain the absence of CICR in rodent muscle. Later, Wang et al. (2005) showed that mechanical stress on the plasma membrane (applied via osmotic changes) elicits ECRE and demonstrated an increased susceptibility to their induction by either osmotic changes or fatiguing exercise in a mouse model of Duchenne muscular dystrophy (*mdx*). This propensity, attributed to reactive oxygen species (Martins et al., 2008) or destabilization of triadic junction by loss of dystrophin (Teichmann et al., 2008), suggested roles of sparks or ECRE in other diseases.

Thus, the operation of CICR was sought in models of malignant hyperthermia (MH), a condition characterized by life-threatening hypermetabolic events mediated by uncontrolled Ca^{2+} release in skeletal muscle and typically associated with gain-of-function mutations in RyR1. Given that hypersensitivity to CICR-promoting caffeine is an MH diagnostic criterion, an enhancement of CICR was expected in MH. This expectation was affirmed by the observation of increased Ca^{2+} sensitivity to activation in isolated human RyRs with MH-linked mutation G2434R (Richter et al., 1997). The outcomes in living cells, however, were largely negative. In a technically heroic comparison of voltage-clamped fiber segments biopsied from 18 patients with either positive (MHS) or negative (MHN) diagnosis (Struk et al., 1998), the V_m-elicited Ca^{2+} release was kinetically similar, differing only for greater amplitude in MHS cells. Likewise, Manno et al. (2013) found neither ECRE nor responses to SLICs in mouse muscle bearing the Y522S mutation, a significant negative finding, as this animal models a human mutation that causes an MH of florid phenotype, including lesions interpreted as resulting from local CICR. The altered response was instead consistent with a reduced susceptibility of RyRs to CDI.

In an unexpected turn, Apostol et al. (2009) showed that ECRE would not be elicited by osmotic changes if mouse myofibers were held depolarized, which inactivates $Ca_V1.1$. The observation thus indicated an agonist role of $Ca_V1.1$, consistent with Apostol's

finding of fewer ECRE in myogenic cell lines devoid of $Ca_V1.1$. Collectively, these studies identified t-tubular membrane deformation as the main determinant of ECRE and evinced that they may result from altered interactions involving multiple couplon members.

The agonist role of $Ca_V1.1$ suggests that ECRE might just be an abnormal version of DICR. Another disease, however, provided clear evidence of the involvement of CICR in ECRE. On myofibers of a mouse model of amyotrophic lateral sclerosis, Zhou et al. (2010) found segments where mitochondria either lost function or disappeared altogether (Fig. 10). In these regions, osmotically elicited ECRE evolved to widespread Ca^{2+} release that stopped sharply at the edge of the failing segment (Fig. 10 B). The observations demonstrated that buffering by mitochondria is crucial for the stable patterning of muscle Ca^{2+} signals. They also helped assert in general that CICR is involved in ECRE.[10]

Collectively, these studies identify local abnormal Ca^{2+} release as a potential contributor to the pathogenic processes linked to mutations in various proteins of the triad. In most cases, however, CICR does not appear to contribute to the altered Ca^{2+} release.

Conclusions and questions

The laboriously reached consensus, illustrated by Fig. 11, is that CICR contributes to EC coupling in skeletal muscle, where it results in the production of Ca^{2+} sparks. Normally, the contribution is limited to muscles containing RyR isoform 3, including many tested muscles of birds, fish, and amphibians. In these muscles, CICR contributes to the fast rise in cell-wide flux and is rapidly terminated by CDI. In most mammalian muscles, CICR is nil under physiological conditions; the waveform of Ca^{2+} flux elicited by step depolarization still shows a peak, however, which is explained by rapid CDI. Most muscles of nonmammalian taxa have one triad per sarcomere; in these, a CICR amplification of DICR flux may be needed to ensure adequate binding of Ca^{2+} to its target sites on troponin, located hundreds of nanometers away from the release sites. The stages of evolution leading to mammals apparently found more liabilities than advantages in CICR, resulting instead in the adoption of a design comprising two exclusively V_m-controlled triads per sarcomere. CICR is present, however, in developing muscle, where it appears limited to regions devoid of t tubules. CICR-mediated local events, ECRE, also appear in adult mammalian muscle under special conditions, including after removal of plasmalemma and dedifferentiation as well as mechanical stress and some diseases. ECRE should be contemplated as a pathogenic mechanism in myopathies that exhibit local cellular damage.

The processes that result in the stoichiometric arrangement of Ca_Vs and RyRs remain mysterious, as do two consequences of this arrangement (Fig. 11, question marks). They are the interdiction of CICR for RyR1 channels (which, in isolation, are sensitive to Ca^{2+}) and the role of C channels (which could be allosteric "slaves" of their coupled neighbors, occasional contributors via highly restricted Ca^{2+} activation, or reserves for replacement or passive spacers). More questions emerge when the often contradictory indications of control of Ca^{2+} release inside cardiac muscle SR are translated to skeletal muscle (Fig. 11; Sobie et al., 2017).

[10]The possibility of comparing signals in segments with and without functional mitochondria within the same cell was later used by Yi et al. (2011) for the first quantitative estimation of the flux of Ca^{2+} removal by mitochondria in EC coupling of skeletal muscle.

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