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CALCIUM SIGNALING BETWEEN SARCOLEMMAL CALCIUM CHANNELS AND RYANODINE RECEPTORS IN HEART CELLS

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1. ABSTRACT

Cardiac excitation-Ca^{2+} release coupling is, in essence, a tale of two molecules, sarcolemmal voltage-gated L-type Ca^{2+} channels (LCCs) and intracellular ryanodine receptors (RyRs), communicating via the Ca^{2+}-induced Ca^{2+} release mechanism. Recent advances have provided a microscopic view of the intermolecular Ca^{2+} signaling between LCCs and RyRs. In a dyadic junction or a "couplon", LCCs open and close stochastically upon depolarization, delivering a train of high local Ca^{2+} pulses ("Ca^{2+} sparklets") to the RyRs in the abutting SR terminal cisternae. Stochastic activation of RyRs discharges "Ca^{2+} sparks" from different couplons, which summate into global Ca^{2+} transients. Hence, ignition of Ca^{2+} sparks by Ca^{2+} sparklets constitute elementary events of EC coupling. While the sparklet-spark coupling is of low fidelity (at 0 mV, about one out of 50 sparklets triggers a spark under physiological conditions), the *high-gain amplification* of CICR (~15 at 0 mV) is achieved because of the greater single-channel flux and open time of RyRs and multi-RyR origin of Ca^{2+} spark. The *global stability* of CICR is safeguarded by many factors acting in synergy, including physical separation of RyR clusters, sheer Ca^{2+} gradients around the channel pores, low intrinsic Ca^{2+} sensitivity of RyRs *in vivo*, and high cooperativity for the Ca^{2+}-dependent spark activation. The *local stability* of CICR is insured because of strong, use-dependent inactivation of RyRs, that terminates Ca^{2+} sparks and confers persistent local SR refractoriness.

2. INTRODUCTION

In heart muscle cells, excitation-contraction (EC) coupling is a cascade of Ca^{2+}-mediated intracellular signal transduction that links membrane depolarization to activation of cell contraction. Its pivotal first step involves crosstalk between two types of Ca^{2+} channels, the voltage-

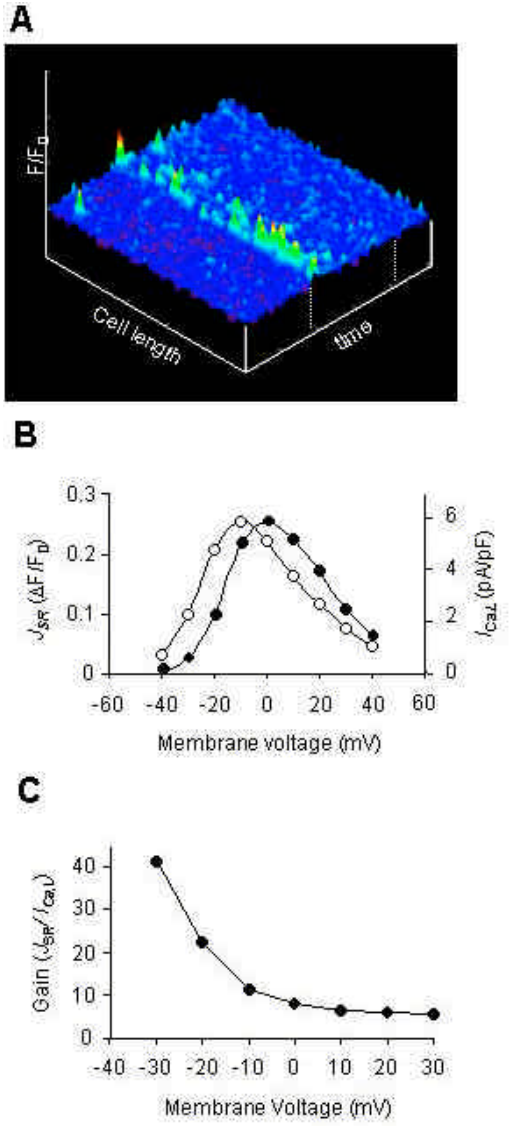

Figure 1. Ca^{2+} spikes and the gain function of EC coupling. Whole-cell patch-clamp was established with the cell dialyzed with 1 mM Oregon Green BAPTA 5N and 4 mM EGTA. *A.* Ca^{2+} spikes elicited by a 300-ms depolarization to –30 mV. Vertical dash lines mark the beginning and end of the voltage pulse, and data are shown as surface plot. *B.* Bell-shaped voltage-dependence for SR Ca^{2+} release flux (J_{SR}, measured by spatially averaged Ca^{2+} spikes) (open symbols) and $I_{Ca,L}$ (solid symbols). Note that the J_{SR} curve is shifted leftward by about 10 mV. *C.* Voltage-dependence of the gain function J_{SR} /$I_{Ca,L}$. (*A:* unpublished data; *B*&*C*: data from reference *27*).

perated L-type Ca^{2+} channels (LCCs) (*1*) in the sarcolemma and the Ca^{2+} release channels/type 2 ryanodine receptors (RyRs) (*2*,*3*) in the sarcoplasmic reticulum (SR). The LCC-to-RyR communication relies on the incoming Ca^{2+} as the second messenger to activate the RyRs via the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (*4*,*5*). Simultaneously simple and enigmatic, CICR has attracted much attention over the last decade, calling for elucidation of its reliability, controllability, and stability. Many advances, including the conception of local control of CICR (*6*,*7*), the discovery of tale-telling microscopic Ca^{2+} events, namely "Ca^{2+} sparks" (*8*), "Ca^{2+} sparklets" (*9*) and "Ca^{2+} spikes" (*10*), as well as the ongoing quest for the mechanism that terminates CICR (*11-14*), have greatly deepened our understanding of this core Ca^{2+} signaling mechanism at the molecular level. This brief review focuses on mechanistic aspects of intermolecular signaling between LCCs and RyRs, to provide a microscopic view of EC coupling. Comprehensive overview of related topics can be found elsewhere (*15-18*) and in companion articles in this issue of *Frontiers in Bioscience*.

3. LOCAL CONTROL OF CICR

The phenomenon of CICR was initially demonstrated in skinned skeletal muscle fibers (*19*,*20*) and cardiac Purkinje cells (*4*,*5*) by abruptly increasing the bathing Ca^{2+} concentration, where the trigger Ca^{2+} comes from the bulk solution surrounding the exposed SR. In his classic and elegant experiments, Fabiato demonstrated that cardiac CICR is graded both by the magnitude and the rate of change (d[Ca]/dt) of the trigger Ca^{2+}, and that supra-optimal trigger Ca^{2+} negatively regulates CICR, resulting in attenuated SR Ca^{2+} release (*5*). These observations have been interpreted by a model in which fast, low affinity Ca^{2+}-dependent activation occurs concurrently with a slow, high-affinity Ca^{2+}-dependent inactivation (*5*).

A contemporary version of the Fabiato experiment has been performed in intact cardiac myocytes, where flash photorelease of caged Ca^{2+} produces a homogenous step increase of cytosolic Ca^{2+}. The photolytic Ca^{2+} suffices to activate the SR Ca^{2+} release, in a similarly graded fashion (*7*,*21*). During normal EC coupling, however, LCC current ($I_{Ca,L}$) serves as the physiological trigger of CICR. Several salient features of the $I_{Ca,L}$-elicited SR Ca^{2+} release have been identified by independent laboratories. These include a bell-shaped voltage dependence that reminisces that for $I_{Ca,L}$ (*22-27*), a high-gain amplification (~10-20 at 0 mV) (*26*,*27*), a voltage-dependent reduction of the "gain" function (*26*,*27*) (Figure 1), and a variable endurance of the release that is controlled by the duration of $I_{Ca,L}$ (22).

To explain properties of the $I_{Ca,L}$-elicited CICR, a parsimonious model implies that Ca^{2+} entry as $I_{Ca,L}$ raises uniformly the cytosolic Ca^{2+} and thereby affects all RyRs equally. However, Stern proved mathematically that such a "common pool" model in the high-gain zone is inherently unstable, resulting in nearly all-or-nothing behavior (*6*). Indeed, Niggli and Lederer found that it is rather inefficient for photoreleased Ca^{2+} (i.e. common pool Ca^{2+}) to trigger SR Ca^{2+} release as compared to the $I_{Ca,L}$ (*7*). These experimental and theoretical reasoning have led to the proposal that RyRs are instead under tight *local control* by LCCs (*6*,*7*). Specifically, co-localization of RyRs and LCCs (*28*) allows the high local Ca^{2+} in the close proximity of open LCCs preferential access to RyRs, which are presumably insensitive to low levels of Ca^{2+}. Stern further envisaged that a single LCC controls either a single RyR

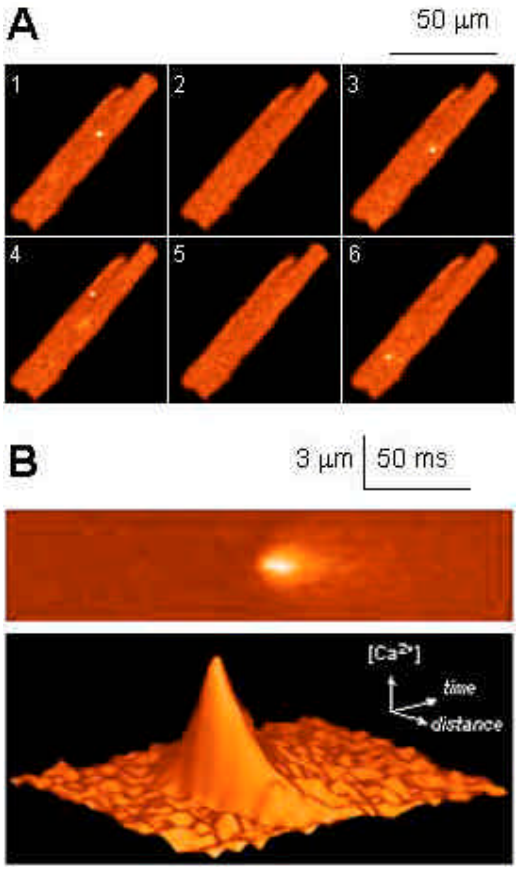

Figure 2. Ca^{2+} sparks in cardiac myocytes. *A.* Six consecutive confocal raster-scan images (1 s apart) of a quiescent ventricular myocyte loaded with the Ca^{2+} indicator, fluo-3. Ca^{2+} sparks are discernible as bright spots occurring randomly inside the cell. *B.* Linescan confocal image of a typical Ca^{2+} spark (top) and its surface plot (bottom), showing the temporal (horizontal) and spatial (vertical) characteristics of the spark. (Unpublished data).

("kiss" model) or a group of RyRs ("cluster bomb" model) (*6*), and individual units operate independently, by virtue of spatial separation and of sheer Ca^{2+} gradients from a point source (*29*). As a result, a high-gain amplification (reliability) can be achieved without jeopardizing the stability and controllability of CICR. This model also explains why an early interruption of $I_{Ca,L}$ abbreviates the SR Ca^{2+} release (*22*).

To evaluate the competing CICR models, Wier et al (*26*) examined the efficiency of $I_{Ca,L}$ to activate SR Ca^{2+} release at different voltages. In the "local control" model, properties of unitary LCC currents (i_{Ca}) is an important determinant, so the SR Ca^{2+} release is not necessarily a unique function of the whole-cell $I_{Ca,L}$. To the contrary, the common pool model predicts that SR release depends solely on the $I_{Ca,L}$. The experimental results revealed that the efficiency of $I_{Ca,L}$ as the trigger is progressively diminished with increasing voltage: comparable $I_{Ca,L}$ triggers greater Ca^{2+} release at more negative voltages, when i_{Ca} and hence local Ca^{2+} pulses are greater. This provides strong indirect evidence in favor of the local control theory.

An independent line of evidence for the local control theory came from investigation of the efficacy of Ca^{2+} influx via routes other than the LCCs. The prediction is that CICR would be less effective if these alternative routes are not as well aligned to RyRs. To this end, Ca^{2+} entry via the reverse mode Na^{+}/Ca^{2+} exchange ($I_{Ca,\ NCX}$) is 20-160 times less effective (*30*), while T-type Ca^{2+} channel current ($I_{Ca,T}$) is also very inefficient in triggering CICR (*31*). Early evidence for localized CICR also includes the observation that local activation of Ca^{2+} transient from one end of a rat cardiac myocyte does not propagate into the remote end of the cell (*32*).

4. Ca^{2+} SPARKS: ELEMENTARY EVENTS OF SR Ca^{2+} RELEASE

"Ca^{2+} sparks" are local, brief and small increases of intracellular Ca^{2+} visualized by confocal microscopy in conjunction with the new generation of fast and high-contrast Ca^{2+} indicators (*8,33*) (Figure 2). Originated from RyRs in the SR, they occur spontaneously in resting myocytes, while identical Ca^{2+} sparks can also be evoked by $I_{Ca,L}$ (*34-38*). These spontaneous Ca^{2+} sparks are independent of LCC Ca^{2+} entry (*8,33,39,40*), are sensitized by low doses of caffeine and ryanodine, and are inhibited by high doses of caffeine or ryanodine as well as Mg^{2+} and tetracaine (*8,39*). During EC coupling, spatial and temporal summation of up to 10^4 Ca^{2+} sparks gives rise to the cell-wide global Ca^{2+} transients (*34-38*). Hence, Ca^{2+} sparks constitute elementary events of cardiac EC coupling. When CICR is somehow tipped to the verge of instability, however, solitary Ca^{2+} sparks no longer remain confined (*41-43*); recruitment of discrete Ca^{2+} sparks often evolves into saltatory propagating waves of Ca^{2+} excitation (*41*), indicating that Ca^{2+} sparks are also elemental to initiation and propagation of Ca^{2+} waves.

The existence of Ca^{2+} sparks immediately told us something that we had not appreciated before. First, SR Ca^{2+} release occurs in a stochastic and discrete manner. Mapping the origin of Ca^{2+} sparks revealed that spark-generating sites, coincident with T-tubules, are separated by ~1.8 in the longitudinal direction and 0.5-1.5 µm in the transverse direction (*6,41,42,44*). Genesis of Ca^{2+} spark at T-SR junctions during small depolarization has been shown to be governed by Poisson statistics (*35*). The concept that CICR is discrete and random is not trivial, because not all properties of a stochastic and discrete system can be described by a deterministic and continual model. From an engineering's standpoint, the discreteness provides a straightforward, yet ingenious, solution to the stability and controllability of CICR: gradedness of CICR can be achieved simply by varying the number of sparks recruited. Izu et al (*45*) has recently noted another intriguing difference between the two classes of models with respect to Ca^{2+} wave initiation and propagation.

Second, the rate of occurrence of spontaneous sparks suggests that RyRs *in situ* are surprisingly

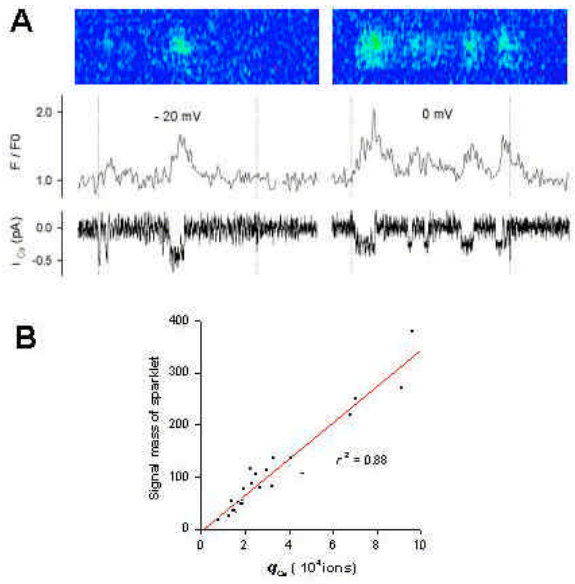

Figure 3. Ca^{2+} sparklets due to single LCC openings. GO-seal patch clamp was established on an intact cardiac myocyte whose SR was paralyzed by 10 mM caffeine and 10 μM thapsigargin. The patch pipette (3-5 MO) contained 10 μM FPL64176 and 20 mM Ca^{2+}. Confocal linescan was focused right beneath the patch membrane. A. Ca^{2+} sparklets during 400-ms depolarization at –20 and 0 mV (top), their line plots (middle), and the simultaneously recorded unitary Ca^{2+} current, i_{Ca} (bottom). B. Linear correlation between sparklet signal mass ($\Pi\Delta F/F_0 dxdt$) (in arbitrary unit) and the integral of the corresponding i_{Ca}, q_{Ca} (number of Ca^{2+} ions). (Data from reference *9*).

insensitive to Ca^{2+}, validating an important premise of the local control theory. Out of ~10^6 RyRs exposed to ~100 nM resting Ca^{2+} in a typical myocyte, approximately 100 Ca^{2+} sparks ignite every second (*8*). This translates into an open frequency of 0.0001 s^{-1} or a mean close time of 10,000 s for RyRs in cells, differing by orders of magnitude from those in the planar lipid bilayer (*46,47*). The low excitability of RyRs in milieu of intact cells should help to confine CICR both in space and time.

Once activated, Ca^{2+} sparks evolve autonomously and reach the peak in ~10 ms, regardless of the turn-off of trigger Ca^{2+} (*35*). The brevity of Ca^{2+} sparks is unexpected, and indicates that regenerative CICR within a spark-generating unit must be somehow terminated promptly (see below). Together, the spontaneous termination, the spatial confinement of Ca^{2+} sparks, and the low Ca^{2+} sensitivity of RyRs provide important bases for the local control theory.

Because of their intracellular location, RyRs in intact cells have thus far defied direct electrophysiological measurement. Confocal imaging of Ca^{2+} sparks has provided a novel and powerful means by which RyR activity *in situ* can be observed non-invasively, on the smallest physiological scale. To date, Ca^{2+} sparks are shown to be present in all types of muscles (*8,48-50*). Ca^{2+} sparks of both RyR and IP_3 receptor (IP_3R) origin are present in non-excitable cells such as glial cells (*51*) and endothelial cells (*52*). Analogous IP_3R Ca^{2+} sparks, named "Ca^{2+} puffs", have also been extensively characterized in xenopus oocytes (*53*). Ca^{2+} sparks as the universal building blocks of Ca^{2+} signaling fulfill distinctly different physiological roles in a cell-type specific manner.

While Ca^{2+} sparks appear to constitute the totality of SR Ca^{2+} release in $I_{Ca,L}$-elicited Ca^{2+} transients, Niggli and Lipp have demonstrated non-spark, spatially uniform Ca^{2+} release when the SR is activated by photolytic Ca^{2+} (*54,55*) or reverse mode Na^+/Ca^{2+} exchange (*56*). They proposed that this type of release is mediated by sub-spark events, termed "Ca^{2+} quarks" (*54*), that are perhaps from single RyRs and are not readily discernible (*17,54-56*). However, if quarks and sparks differ only in the number of RyRs involved, it is difficult to reconcile why Ca^{2+} entry other than $I_{Ca,L}$ activates only the subtler events, whereas resting Ca^{2+} activates SR release in the form of spontaneous Ca^{2+} sparks. The condition, extent and physiological relevance of non-spark SR Ca^{2+} release merits further investigation.

5. TRIGGER Ca^{2+} ENTRY: VISUALIZATION OF Ca^{2+} SPARKLETS FROM SINGLE LCCs

In a microscopic perspective, Ca^{2+} entry gated by single LCC openings ought to be discontinuous and stochastic as well. A typical i_{Ca} at 0 mV is of ~0.12 pA (2 mM Ca^{2+} as the charge carrier) (*57*) and lasts ~0.3 ms (*58*), carrying a packet of Ca^{2+} of ~110 ions. This tiny amount of Ca^2 is beyond the detection limit of current generation of confocal fluorescent microscopy. Nevertheless, when i_{Ca} is prolonged and enlarged by the LCC agonist FPL64176 and 10-20 mM external Ca^{2+}, we were able to visualize Ca^{2+} entry from single LCCs, dubbed "Ca^{2+} sparklets", in cells whose SR Ca^{2+} release was paralyzed (*9*) (Figure 3). The onset and offset of a Ca^{2+} sparklet follow closely the open and closure of the channel; the fluorescent "signal mass" of Ca^{2+} sparklets linearly correlates with the integral of the corresponding i_{Ca} (8,000 to 100,000 Ca^{2+} ions) (Figure 3). Thus, in addition to Ca^{2+} sparks, Ca^{2+} sparklets afford another tool for investigation of microscopic properties of LCC-to-RyR coupling. In circumstances when it is impossible to record i_{Ca} electrophysiologically, Ca^{2+} sparklets as an optical readout of i_{Ca} can be exploited to monitor gating of single LCCs (*9*). Moreover, Ca^{2+} sparklets produced by a known size of i_{Ca} may serve as an optical standard to calibrate Ca^{2+} release flux underlying a spark (*9*). This calibration is basically model-independent and parameter-free, because sparklets and sparks share the common microenvironments with respect to indicator binding, Ca^{2+} buffering and diffusion.

6. TRIGGERING Ca^{2+} SPARKS BY SINGLE LCC EXCITATION

The ultimate test for the local control theory would be the demonstration that single LCC excitation triggers discrete SR Ca^{2+} release events, or Ca^{2+} sparks. Two independent groups provided the first supporting evidence. Cannell et al (*35*) noticed that the voltage-dependence of Ca^{2+} spark activation (P_s) is proportional to

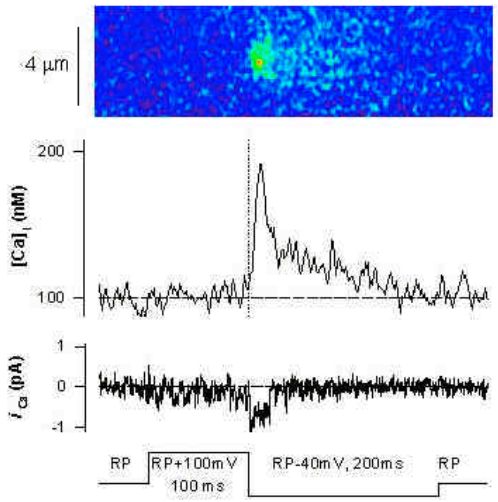

Figure 4. Triggering Ca^{2+} spark by single LCC excitation. The experimental conditions were the same as in Figure 2, except that the cell was bathed in normal physiological saline. A Ca^{2+} spark is evoked by tail i_{Ca} upon hyperpolarization. RP: resting potential, ~-70 mV in rat ventricular myocytes. (From reference *9*).

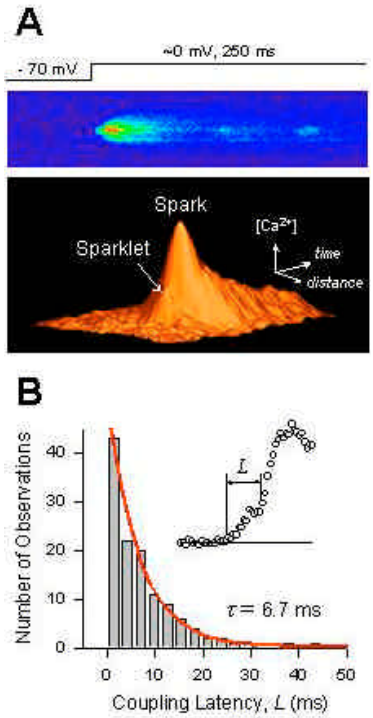

Figure 5. Sparklet-spark coupling under loose-seal patch clamp conditions. The patch pipette (3-5 MΩ) was gently pressed against the cell membrane to form low-resistance seal (30~50 MΩ) without suction. Other experimental conditions were the same as in Fig. 4. A. Representative example showing that a Ca^{2+} sparklet of LCC origin directly triggers a Ca^{2+} spark. Note that two additional Ca^{2+} sparklets in the wake of the spark fail to trigger further SR Ca^{2+} release, suggestive of local refractoriness. B. Histogram distribution of the sparklet-spark coupling latency (*L*, insert). Monoexpoenential fitting yields a time constant τ= 6.7 ms. (Data from reference *9*).

LCC activation ($P_{o,L}$) at near-threshold voltages (from -60 to -40mV), displaying an *e*-fold increment per ~7 mV depolarization. Under conditions when most LCCs are inhibited (for resolution of solitary sparks), López-López et al (*36*) demonstrated kinetic similarities between the residual $I_{Ca,L}$ and the latency distribution of spark activation over a wide range of voltages. Both lines of evidence indicate that spark activation does not require cooperative interaction among LCCs, because, otherwise, P_s would be expected to be a power function of $P_{o,L}$, or P_s % $P_{o,L}^{x}$, with $x > 1$.

To directly demonstrate that a single opening of an LCC triggers a Ca^{2+} spark, it is crucial to record i_{Ca} and its triggered spark simultaneously. Ever since the first recording of evoked Ca^{2+} sparks, this has been a formidable task attempted by many laboratories (*9*,*59*). With the development of a novel method to combine GΩ-seal single-channel patch clamp and confocal spark detection in our laboratory, we visualized Ca^{2+} sparks activated beneath the patch membrane by single LCC openings (*9*) (Figure 4). Further, to avoid the "Ω"-shaped membrane deformation associated with GΩ-seal patch clamping, which disrupts the delicate LCC-to-RyR coupling on most occasions (*9*), we adopted the so-called loose-seal patch-clamp technique (*9*) (Figure 5). In the presence of FPL64176, both high- and low-amplitude local Ca^{2+} events are evoked in loose patches responding to depolarization to ~ 0 mV. The low-amplitude events are ryanodine-resistant, representing Ca^{2+} sparklets, whereas the high-amplitude events represent Ca^{2+} sparks. Fig 5 shows a Ca^{2+} spark rising from the shoulder of an ongoing Ca^{2+} sparklet. These results not only validate a prescient prediction by Stern (*6*), but also provide the first real-time recording of Ca^{2+} signal transduction at the single-molecule resolution.

7. FIDELITY, KINETICS AND STOICHIOMETRY OF LCC-TO-RyR COUPLING

With the advances in ultrastructural and functional studies, a microscopic view of cardiac EC coupling emerges. Dozens of LCCs, up to a few hundreds of RyRs, along with their accessory and regulatory proteins, co-localize to dyadic junctions where the surface membrane and transverse tubules come within 15 nm of the SR membrane (*28*,*60*). A structural, functional and regulatory unit of EC coupling, or a "couplon" (*61*), encompasses the two membrane systems, the junctional cleft, and the molecules wherein. LCCs open and close stochastically upon depolarization, delivering a train of trigger Ca^{2+} pulses or Ca^{2+} sparklets, to the RyRs in the abutting SR terminal cisternae. Stochastic activation of RyRs discharges Ca^{2+} sparks from different couplons, which summate into global Ca^{2+} transients. Hence, from a reductionism's perspective, to understand the EC coupling and its physiological regulation is, in essence, to unravel the secrets concealed within the nanoscale couplon.

The ability to observe Ca^{2+} sparklets directly igniting Ca^{2+} sparks (Figure 5) permitted us to determine the kinetics of LCC-to-RyR communication. By measuring the temporal length from the onset of a sparklet to the onset

of its triggered spark, we obtained the latency of LCC-to-RyR coupling in intact cells at ~0 mV in the presence of FPL64176 and high external Ca^{2+}. Its histogram distribution is well described by a single-exponential function, with a time constant of 6.7 ms (*9*) (Figure 5). Because of stochastic variations of both LCC open time and spark activation, not every LCC opening is expected to trigger a Ca^{2+} spark. The LCC-to-RyR coupling fidelity (*d*), i.e., the fraction of LCC openings that successfully trigger sparks, was determined to be d . 0.7 under our experimental conditions (*9*).

For EC coupling under normal conditions, a d value of 0.02 has been estimated based on macroscopic gain function and microscopic properties of i_{Ca} (*62*). In other words, on average, one out of ~50 LCC openings at 0 mV triggers a Ca^{2+} spark under normal conditions. This is in general agreement with the results of single RyRs reconstituted in planar lipid bilayers, where photoreleased Ca^{2+} of 9 μM and 0.1-0.4 ms activates the channel with d . 0.06 (*63*). Despite the low coupling fidelity, a high-gain amplification is realized because of the large single-channel flux and long open time of RyRs, and likely, multi-RyR activation in a spark (see below).

8. NONLINEAR Ca^{2+}-DEPENDENCE OF Ca^{2+} SPARK ACTIVATION

The efficacy of $I_{Ca,L}$ as the trigger of SR Ca^{2+} release or the "gain" function is a tale-telling quantity of EC coupling, and has been measured in different experimental settings. Under whole-cell voltage-clamp conditions, the "gain" is usually defined as the ratio between peak SR Ca^{2+} release flux (J_{SR}) and peak $I_{Ca,L}$, where the J_{SR} is derived from global Ca^{2+} transients with the aid of mathematical modeling (*26,64*), or directly measured using a combination of a fast, low-affinity Ca^{2+} indicator (such as Oregon green BAPTA 5N) and a slow, high-affinity nonfluorescent Ca^{2+} chelator (such as EGTA) (*10,27*). When solitary, evoked Ca^{2+} sparks can be counted (e.g., in the presence of LCC antagonist), the gain function can also be defined as the ratio between P_s and $I_{Ca,L}$ (*65*). All measurements indicate that the gain function decays with increasing voltage. Importantly, Santana et al (*65*) noticed that the voltage-dependence of gain function essentially overlaps the voltage-dependence of i_{Ca} predicted from the Nernst-Planck relationship. This result has been interpreted to reflect that spark activation depends on the square of intra-couplon Ca^{2+} concentration (which should be proportional to i_{Ca} (*66*)), or P_s % i_{Ca}^2 % $[Ca^{2+}]^2$(*65*). Highly cooperative Ca^{2+}-dependent activation has also been observed *in vitro* for RyRs in response to Ca^{2+} steps (*46,47*) or Ca^{2+} pulses (*63*), indicating that it is likely an intrinsic property of the RyR.

The high cooperativity for Ca^{2+}-dependent activation appears to be one of the most important features of cardiac EC coupling. Owing to the steep power relationship, RyRs, which are essentially insensitive to resting Ca^{2+} (0.1 μM), can robustly respond to i_{Ca}-produced local Ca^{2+} pulses (several tens μM) (*66*). It also confers the ability for RyRs to discriminate local trigger Ca^{2+} against spatially averaged Ca^{2+} (μM) during an ongoing Ca^{2+} transient.

9. HOW MANY RyRs ACTIVATED IN A SPARK?

The exact *Nature* of Ca^{2+} sparks remains elusive. Initial evidence was ambivalent as to single- or multi-channel origin of Ca^{2+} sparks (*8*). Evidence in favor of single-channel origin of sparks has been twofold. First, low concentration of ryanodine (*8*) or FK506 (*13,14*) favor the appearance of long-lasting sparks with halved amplitude, akin to subconductance states of RyR in planar lipid bilayer in the presence of the channel ligand. This interpretation is now known to be flawed because plateau amplitude at 50% height implies a Ca^{2+} flux several times smaller than that generating the peak (*67*). Second, the estimated Ca^{2+} flux underlying a spark (i_{spark}, 2~4 pA) (*8,68*) was close to unitary Ca^{2+} current of the RyR measured with 10 mM Ca^{2+} at 0 mV (*69,70*). However, more recent *in vitro* measurement under quasi-physiological ionic conditions (inclusion of 1 mM Mg^{2+}) has revised it downward to ~0.35-0.6 pA (*71*). Based on the new estimate of unitary RyR current and sparklet-calibrated i_{spark} (2.1 pA) (*9*), we suggested that a Ca^{2+} spark consists of ~4-6 RyRs in (*9*), similar to what was proposed for Ca^{2+} sparks in skeletal fibers (*67*, see also *72*).

While virtually all numerical models using 1-3 pA i_{spark} can reproduce amplitude and temporal characteristics of sparks, the predicted width (1-μm) is only about half of the value observed experimentally (2-μm) (*73-75*). On one extreme, Izu et al (*76*) proposed that 10-20-pA i_{spark} is required to resolve the "spark-width paradox" (*74*). This large i_{spark} would place the number of RyRs in a spark in the neighborhood of 20-40. Unfortunately, this prediction is somewhat compromised because model construction assumed properties of cytosolic Ca^{2+} buffering and diffusion that are not well known presently.

Much work has focused on spark amplitude as an index of RyR open time and of total Ca^{2+} discharged in a spark. Theory (*77,78*) and numerical analysis (*73,74*) predicted that *apparent* Ca^{2+} spark amplitudes recorded by confocal microscopy should always display a monotonic decaying distribution, regardless of their *true* amplitude distribution. Experimental measurements with the aid of an automated detection algorithm (*77*) have confirmed it. However, Ca^{2+} sparks evoked at fixed positions (*79*) or occurring spontaneously at hyperactive sites (*80*) demonstrated rather stereotyped amplitude. Most recently, we have shown that Ca^{2+} sparks evoked beneath the patch membrane, free of out-of-focus blurring, exhibit a broad modal amplitude distribution (*9*). The modality of the spark amplitude distribution was initially interpreted to reflect a multi-RyR origin of sparks, for single channels are expected to have exponentially-distributed open time, and so are the amplitudes of sparks of single-RyR origin (*79*). Alternatively, the modality could be a manifestation of irreversible gating of a single RyR (*78,81*) (see below).

Taken together, increasing evidence strongly suggests that sparks originate from multiple, instead of single, RyRs; but no study is conclusive as to the exact number of RyRs involved. Interestingly, even for the

wildest estimate, RyRs in a spark encompass only a minor fraction of total RyRs in a couplon (100-200 in rat and mouse) (*28*). So, an outstanding question is why the opening of a few RyRs in a couplon does not fire all RyRs therein? This observation perhaps calls for new, nanoscopic "local control" models to address the molecule-to-molecule crosstalk in a couplon.

10. THERMODYNAMICALLY IRREVERSIBLE GATING OF RyRs *IN VIVO*

For a single or a group of Markov channels gating reversibly, distributions of open and closed times should be the sum of positively weighted decaying exponentials. Violation of this microscopic reversibility has been demonstrated previously on a number of occasions at the single channel level (*82-84*), and has been attributed to possible channel coupling to external sources of free energy. Like the vast majority of ionic channels, single RyRs *in vitro* have been described by Markovian models (*68,70,85-87*) in which transition between discrete conformational states is determined solely by the present state of the channel, independent of history (only beyond 10-20 transitions (*85*)). When such a channel is unperturbed, i.e., uncoupled from an external source of energy, thermodynamic laws require microscopic reversibility of the channel reaction. This means that, at equilibrium, a cyclic reaction must take place at the same rate in forward and backward directions; the stochastic properties of the channel must show time reversibility; and distributions of statistical quantities, such as open time, closed time, and burst time, must each equal a sum of positively weighted, decaying exponential terms (*88*). The same conclusions hold true for a cluster of inter-linked channels that are uncoupled to an external energy source, because such cluster as a whole can be treated as a Markovian entity.

The dual role of Ca^{2+} as both a permeating ion and a regulator of RyR channel (*89,90*) creates the intriguing possibility that RyR gating might be coupled to the free energy in Ca^{2+} electrochemical gradients across the SR. If this were the case, RyRs in intact cells (or in bilayers under asymmetric Ca^{2+} electrochemical potentials) might be expected to gate irreversibly. By measuring release duration of Ca^{2+} sparks (as duration of spontaneous Ca^{2+} spikes), we found that distribution of the release duration exhibits a prominent mode at around 8 ms (*80*). Analysis of the cycle time for repetitive sparks at hyperactive sites revealed a lack of intervals briefer than ~35 ms and a mode at around 90 ms (*80*). These results provide the first clue that Ca^{2+} sparks are generated by thermodynamically *irreversible* stochastic processes. In a sense, a single RyR or, more exactly, a couplon can be considered as the tiniest molecular "clock" that displays somewhat ordered temporal behavior in spite of thermodynamic fluctuations.

Because data from cardiac and skeletal RyRs in planar lipid bilayers with asymmetric *cis* and *trans* Ca^{2+} were consistent with *reversible* gating at the single channel level (*80,89,90*), the irreversibility for Ca^{2+} spark genesis may reside at a supra-molecular level. For instance, CICR and Ca^{2+}-induced inactivation among adjacent RyRs may couple the free energy in the SR transmembrane Ca^{2+} gradients to RyR gating *in situ*, shaping up the unique temporal characteristics of Ca^{2+} sparks. The stereotyped Ca^{2+} spark duration also illustrates how collective RyR gating *in vivo* differs qualitatively from single RyR gating *in vitro*.

11. TERMINATION OF LOCAL Ca^{2+} RELEASE

As discussed above, the *global stability* of CICR is safeguarded by many factors acting in synergy, including physical separation of RyR clusters, rapid decay of Ca^{2+} gradients from a point source in the heavily buffered cytoplasm, low intrinsic Ca^{2+} sensitivity of RyRs *in vivo*, and non-linear Ca^{2+}-dependence of spark activation. However, gradedness of CICR requires also *local stability* of CICR, i.e., the turn off of release in a single couplon. If the inherent positive feedback were not counteracted, CICR within the release units should result in everlasting Ca^{2+} sparks. Several mechanisms have been proposed for the termination of SR Ca^{2+} release. (i) *Ca^{2+}-dependent inactivation* (*5*). Binding of released Ca^{2+} to a high affinity site of RyRs inactivates the channels and shuts off Ca^{2+} release. (ii) *Adaptation of RyRs* (*46,47,86*). The open probability of RyR channels in lipid-bilayers declines spontaneously after activation by a step increase in $[Ca^{2+}]$. The "adapted" state differs from the inactivated state as it retains the responsiveness to subsequent higher $[Ca^{2+}]$ steps. (iii) *Stochastic attrition* (*6*). Simultaneous stochastic closing of RyRs in an active couplon results in rapid dissipation of local $[Ca^{2+}]$ and thereby interruption of the positive feedback. In addition, *local SR Ca^{2+} depletion* may also extinguish Ca^{2+} release due to the lack of releasable Ca^{2+} or reduction in the gain of CICR (*39,91,92*, see also *93*).

Test of Ca^{2+}-dependent inactivation in intact cardiac myocytes was first attempted by Nabauer and Morad (*21*). They showed that a mild to moderate photolytic elevation of cytoplasmic Ca^{2+} does not prevent the SR from subsequent activation by $I_{Ca,L}$, though the magnitude of release is apparently attenuated. This has been interpreted as evidence against Ca^{2+}-dependent inactivation of RyRs *in situ* (*21*). Yasui et al (*94*) demonstrated that depolarization to +30 mV in the presence of FPL64176 elicits a transient Ca^{2+} release that terminates despite continued $I_{Ca,L}$. Yet, additional Ca^{2+} release can be triggered by tail $I_{Ca,L}$ upon repolarization. This has been interpreted as evidence for RyR adaptation (*94*). By direct measurement of local SR Ca^{2+} release fluxes ("Ca^{2+} spikes") at individual T-SR junctions, we found that that the tail $I_{Ca,L}$-elicited Ca^{2+} spikes are most likely originated from RyRs unfired during depolarization, rather than from those in the adapted state (*11*). Furthermore, increasing the open duration and promoting the reopening of LCCs with FPL64176 does not prolong or trigger secondary Ca^{2+} spikes. At 50 ms after a maximal release, a multi-fold increase in i_{Ca} (by hyperpolarization to –120 mV) fails to evoke any additional release, indicating absolute refractoriness of RyRs (*11*). These results supports the

notion that Ca^{2+} release is terminated primarily by a strong, local, and use-dependent inactivation of RyRs, and argues against the stochastic closing and adaptation of RyRs as major termination mechanisms of SR Ca^{2+} release in intact cardiac myocytes.

12. LOCAL REFRACTORINESS OF SR CA^{2+} RELEASE

Recently, DelPrincipe et al (*12*) reported that, unlike global SR Ca^{2+} release in response to homogenous flash, focal photolytic Ca^{2+} pulses at 300-ms intervals activate local SR Ca^{2+} releases that do not undergo refractoriness whatsoever. They argued that global refractoriness might be due to SR Ca^{2+} depletion, whereas the lack of local SR refractoriness is due to rapid replenishment during local excitation. However, the apparent lack of RyR inactivation in these experiments can be explained by several other possibilities, e.g., recruitment of different RyRs in consecutive pulses, recovery of inactivated RyRs (absolute refractoriness in the Ca^{2+} spike experiment was detected within 50 ms (*11*)), and overload of local SR with the exogenous photolytic Ca^{2+}.

Using the loose-seal patch clamping and confocal imaging technique, we have revisited the issue of local SR refractoriness by activating single couplons with single LCC excitation (*9*). We found that repetitive Ca^{2+} sparklets can trigger more than one Ca^{2+} spark during a single voltage step. However, the sparklet-spark coupling fidelity, δ, decreases from 0.7 to 0.3 once a spark has being fired. This observation provides direct evidence that RyRs display use-dependent inactivation at the single couplon level. The robust termination of Ca^{2+} sparks and persistent refractoriness add to the repertoire of safekeeping mechanisms that insure the stability and controllability of CICR.

13. PERSPECTIVE

From the conception of local control of CICR, to the first recording of Ca^{2+} sparks, to the demonstration of ignition of Ca^{2+} sparks by single LCCs, and to the search for possible termination mechanisms, our understanding of the physiological processes of EC coupling has been greatly advanced since the discovery of CICR. The emergence of a microscopic picture of LCC-to-RyR communication, along with the advent of novel investigative tools, allows one to define normal, altered and dysfunctional EC coupling with unprecedented accuracy. As the frontier expands, many challenging questions remain open. The following presents our perspective of the six most enigmatic issues in this field.

13.1. Role of SR lumenal Ca^{2+} in regulation of CICR.

It is generally accepted that increasing the SR Ca^{2+} content beyond a critical level greatly enhances RyR sensitivity to induce unstable CICR (*95*). The converse effect, i.e. whether decreasing SR Ca^{2+} negatively regulates RyR gating and thereby terminates the SR Ca^{2+} release, remains controversial. On one hand, we observed that up to 60% depletion of SR Ca^{2+} has no significant effect on the rate of occurrence of spontaneous Ca^{2+} sparks, when the low-amplitude missing events are accounted for (*93*). On the other hand, in chemically skinned cardiac myocytes whose SR Ca^{2+} is primed to a supra-normal level, altering the SR Ca^{2+} load does change Ca^{2+} spark frequency after correction (*39*). At the cellular level, while it has been reported that reduction of SR Ca^{2+} content reduces proportionally the $I_{Ca,L}$-elicited Ca^{2+} release (*96*), Shannon et al (*91*) showed that ~50% depletion of SR Ca^{2+} completely abolishes the RyR response to the trigger $I_{Ca,L}$. *In vitro* experiments also yielded contradicting results. Gyorke and Gyorke (*92*) have shown that a 250-fold reduction of lumenal Ca^{2+} (from 5 mM to 20 μM) shifts the Ca^{2+}-dependent activation of RyR rightward (0.57 logarithmic unit) and downward (by 60%); however, Meissner and colleagues have shown that permeating Ca^{2+} acting at cytosolic RyR sites may pose as lumenal Ca^{2+}-mediated regulation (*89,90*). Future experiments are warranted to determine the condition, extent and physiological relevance of allosteric modulation of the RyR by SR lumenal Ca^{2+}.

13.2. *Nature* of SR Refractoriness

While use-dependent inactivation of RyRs has been established at the cellular (*11, 12,41*), T-SR junctional (*11*), and single-couplon (*9*) levels, it is unclear whether it is a manifestation of Ca^{2+}-dependent negative feedback control mechanism, or a fateful consequence of channel activation *per se*. Moreover, lumenal Ca^{2+} might play a role if a partial depletion affects RyR sensitivity to cytosolic Ca^{2+}.

13.3. Intermolecular communication in a couplon

This utterly important yet difficult issue is yet to be approached. How many RyRs crosstalk to a given LCC, and how many LCCs to a given RyR? Do a single LCC communicate privately to its nearest neighbors, or promiscuously to all RyRs in a couplon? Do RyRs communicate via CICR, or be coupled mechanically by FK506 binding proteins (FKBP) (*97*), or both? If a mechanical coupling is engaged, is it rigid or dynamic in *Nature*? It is imperative to explain what prevents the entire couplon encompassing ~150 RyRs from firing all at once in a spark.

13.4. Gating scheme for RyRs *in vivo*

For various gating schemes derived from *in vitro* experiments, none could reproduce essential features of cardiac EC coupling when implanted into a stochastic couplon model of EC coupling (*61*). Future research should aim also at elucidating the physiologically relevant gating scheme of the channel. At present, we have only a glimpse of some key features involved: a rapid, highly cooperative Ca^{2+}-dependent activation; a profound, and enduring inactivation; and a mechanism that produces preferred active time of the couplon. It should also be emphasized that the collective behavior of a group of RyRs may differ quantitatively and qualitatively from a RyR acting *solo* (*61,80*).

13.5. Regulatory role of signaling molecules complexed with RyRs

Gating of RyRs in intact cells could be even more complex than we thought, for the native channel protein is

associated with numerous other proteins, such as calmodulin (*98*), protein kinases (protein kinase A, calmodulin kinase II) and phosphatases (PP1 and PP2a) (*99*) to form a macromolecular signaling complex. In addition, accessory proteins including FKBP and sorcin may also play functional roles (*13,14,100, 101*). Critical examination of the involvement of these possible regulatory mechanisms in activation, termination and refractoriness of Ca^{2+} sparks calls for future studies.

13.6. Molecular definition of altered or dysfunctional EC coupling

Recent advances have provided an array of novel concepts and microscopic readouts of EC coupling to better define altered or dysfunctional states of EC coupling, as in diseased hearts, in the physiological process of cardiac senescence, or in genetically engineered hearts. For apparently similar phenotypes at the cellular level ($I_{Ca,L}$, J_{SR}, global Ca^{2+} transients, SR Ca^{2+} load, and "gain" function), the underlying microscopic mechanisms may differ (i_{Ca} and sparklets; amplitude, duration and width of Ca^{2+} sparks; temporal synchrony of Ca^{2+} spikes; fidelity and latency of sparklet-to-spark coupling). Insights gained from these studies will certainly enhance our understanding how the heart works in health and in disease.

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