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CALCIUM QUARKS

Ernst Niggli, and Marcel Egger

Department of Physiology, University of Bern, Bühlplatz 5, 3012 Bern, Switzerland

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1. ABSTRACT

Elementary subcellular Ca^{2+} signals arising from the opening of single ion channels may offer the possibility to examine the stochastic behavior and the microscopic chemical reaction rates of these channel proteins in their natural environment. Such an analysis can yield detailed information about the molecular function that cannot be derived from recordings obtained from an ensemble of channels. In this review, we summarize experimental evidence suggesting that Ca^{2+} sparks, elementary Ca^{2+} signaling events of cardiac and skeletal muscle excitation contraction coupling, may be comprised of a number of smaller Ca^{2+} signaling events, the Ca^{2+} quarks.

2. INTRODUCTION

When Ca^{2+} sparks were first identified as elementary events underlying cardiac Ca^{2+} signals (1-2) everyone working in the field of cardiac excitation-contraction (EC) coupling and Ca^{2+} signaling immediately became excited by the perspectives offered by these new findings. Besides having a strong impact on the conception of cardiac excitation-contraction coupling, this discovery was also expected to have implications comparable to those of the first description of ionic currents carried by single membrane channel molecules. Recordings of ion currents on the level of single channels by means of the patch-clamp technique had not only revolutionized the entire field of electrophysiology, but had also dramatically broadened our knowledge about the functioning of channel proteins (for review see 3). Ca^{2+} sparks were thus foreseen to enable us to extract information about single Ca^{2+} release channels *in-vivo*. Furthermore, it was anticipated that similar elementary Ca^{2+} events might underlie Ca^{2+} signaling in cells other than cardiac muscle (for review see 4). Indeed, it became rapidly clear that analogous Ca^{2+} signaling events could be found in many other excitable and unexcitable cells, such as skeletal muscle (5-6) neuronal cells, but also in a variety of unexcitable cells (for reviews see 4, 7-8). After all, scientists not working in the field of Ca^{2+} signaling might wonder why exactly everyone was exceedingly excited about these signals. In the present review, we try to highlight the possible impact of elementary Ca^{2+} signaling events on our understanding of Ca^{2+} signaling.

2.1. Why is the elementary nature of Ca^{2+} signaling important?

Until recently, several important features of cardiac Ca^{2+} signaling were poorly understood on the cellular level. A prominent example is called the “paradox of cardiac Ca^{2+} signaling”. After the seminal studies carried out in the eighties by Fabiato and coworkers (9), it became

generally accepted that in cardiac muscle a small trigger signal mediated by L-type Ca^{2+} current is amplified several-fold by the mechanism of Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR). One might wonder what exactly would be paradoxical with this notion. It can be appreciated intuitively, that the output signal of this amplification, an elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), is identical to the signal that triggers it. Thus, a high degree of positive feedback in CICR and a tendency for instability would be expected. Indeed, this behavior surfaced in attempts to develop computer models of cardiac Ca^{2+} signaling. As it turned out, the experimentally observed degree of amplification could not be simulated without compromising the stability of the model system, at least when "trigger Ca^{2+}" and "released Ca^{2+}" occupied a common cytosolic Ca^{2+} pool. Such models were referred to as "common pool models" (10). However, when CICR was modeled to occur via independent or loosely coupled functional units, each comprising a single L-type Ca^{2+} channel and one ("Ca^{2+} synapse") or several ("cluster-bomb") SR Ca^{2+} release channels, the necessary amplification could be simulated without a tendency for all-or-none behavior. With such a model, regulation of Ca^{2+} release then became easily feasible by recruiting more or fewer functional Ca^{2+} release units. Thus, the apparent paradox was the discrepancy between the required amplification of CICR contrasting with both the experimentally observed maintained control over Ca^{2+} release and the mathematical model predictions. This paradox has been solved by implementing a Ca^{2+} signaling system which is based on the recruitment of functionally independent Ca^{2+} sparks, each spark itself exhibiting the necessary amplification and a high degree of positive feed-back, essentially representing an all-or none event.

2.2. Why is the precise number of channels participating in the generation of Ca^{2+} sparks important?

As mentioned above, the tremendous power of single ion channel analysis has provided insight into features of ion channel function that are not accessible otherwise. For example, with ensemble currents the analysis of microscopic channel gating kinetics and single channel conductance, possibly including some subconductance states, would not have been possible. Unfortunately, for single channels that are located in membranes of intracellular organelles, such as in the SR, the nucleus or mitochondria, the patch-clamp technique cannot be used easily (but see, for example, (11). However, several groups have developed methods to perform similar experimental recordings after reconstituting purified channel preparations (such as SR vesicles) into artificial lipid bilayer membranes. This powerful approach has allowed detailed studies of many aspects of RyR function and resulted in a large body of literature. There is, however, a problem common to all experimental studies carried out in the lipid bilayer system: the channels cannot be analyzed in their native environment. For example, during purification and reconstitution small accessory SR proteins with important functions may be lost. In addition, the composition of the solutions used to examine the channels in lipid bilayers is frequently quite different from the normal cytosol. Because of these difficulties, data obtained from reconstituted channels cannot simply be extrapolated to the behavior of the channels *in-vivo*. This word of caution is further supported by the results of recent attempts to examine the single channel conductance of the RyR in more physiological solutions (12). These experiments again emphasized, that the environment of the channels is extremely important for their normal functioning. From these considerations, it seems obvious that every method which allows to examine the RyRs on the single channel level *in-situ* would be highly valuable, such as imaging of Ca^{2+} sparks in living cells with fluorescent Ca^{2+} indicators. But when we intend to perform studies on Ca^{2+} sparks using methods borrowed from single-channel current analysis, we need to know the number of channels contributing to the generation of a single Ca^{2+} spark.

If we assume, for a moment, that opening of more than one RyR underlies a Ca^{2+} spark, then the situation would be much more complex and an assortment of additional questions would need to be answered to understand Ca^{2+} signaling on this molecular level. For example, we would need to understand how Ca^{2+} flux into the diadic cleft via a single RyR interacts with the remaining neighboring RyRs within a cluster. Can a RyR open independently or does the first opening ignite the entire cluster, all channels synchronized by submicroscopic Ca^{2+}-induced activation or by some allosteric interaction between the densely packed channel proteins (13)? If a single channel or a fraction of the channels within a cluster can open without igniting all of them, we need to identify the as of yet unknown mechanisms that makes some of the channels insensitive for activation. Related to this, it is essential to determine which mechanism (or which mechanisms) terminates the Ca^{2+} release on the level of a single RyR and on the level of a cluster of RyRs. Taken together, in order to understand the activation, regulation and termination of EC-coupling and Ca^{2+} signaling from the molecule to the cell and organ, it is important to have a complete picture of how the involved channels and other proteins talk to each other. This will be even more important when, based on such information, novel pharmacological strategies should be developed for the rational treatment of cardiac conditions in which the reliability and efficiency of EC-coupling are compromised.

3. DEFINITION OF A Ca^{2+} QUARK

Before discussing the experimental results that led us to propose the existence of a Ca^{2+} signaling event that is considerably smaller than a Ca^{2+} spark, termed a "Ca^{2+} quark", it is appropriate to state how such an event is defined. In the past, there has been some confusion in the literature regarding the precise definition of a Ca^{2+} quark. A clear perception of this event is even more important, because the initial proposal of the existence of such events was based on a negative result, as detailed below (14). By definition, a Ca^{2+} quark is the localized subcellular Ca^{2+} signal resulting from the opening of a single SR Ca^{2+} release channel.

4. SR Ca^{2+} RELEASE ACTIVATED BY UV-FLASH PHOTOLYSIS

Spontaneous Ca^{2+} sparks in cardiac muscle cells can be considered as accidents of Ca^{2+} signaling which occur because, at the normal resting $[Ca^{2+}]_i$ of about 100 nM, the probability for a RyR to open is not zero. Very early after the discovery of Ca^{2+} sparks, it was proposed that signals identical to these spontaneous Ca^{2+} sparks might also underlie the Ca^{2+} release during EC-coupling (1). Indeed, this hypothesis was rapidly confirmed in experiments where the number of L-type Ca^{2+} channels activated during a depolarization had been reduced with specifically designed voltage-clamp protocols or pharmacologically (15-16), see also figure 1A. Both strategies revealed that the normal Ca^{2+} transient was in fact composed of a large number of synchronized Ca^{2+} sparks, each exhibiting properties similar to the spontaneous Ca^{2+} sparks. After these studies were published, many were wondering whether the resemblance of spontaneous and triggered Ca^{2+} sparks was a pure coincidence or whether an identical functional SR Ca^{2+} release unit was opening in either case (possibly corresponding to a single RyR channel). Alternatively, the size of a Ca^{2+} spark might have been determined by the trigger signal, the opening of L-type Ca^{2+} channels. But this seemed less likely, because Ca^{2+} influx via L-type Ca^{2+} channels was not thought to be necessary for the incidents of spontaneous Ca^{2+} sparks (but see 17). To address these questions, we decided to embark on a study in which we did not need to rely on L-type Ca^{2+} channels to trigger Ca^{2+} sparks. Instead, we decided to use flash-photolysis of caged Ca^{2+} to provoke SR Ca^{2+} release, while simultaneously imaging the subsequent Ca^{2+} release events with laser-scanning confocal microscopy (figure 1B and C). At photolytic power levels designed to activate only a small number of functional SR Ca^{2+} release sites, we should be able to spatially resolve individual Ca^{2+} release events. Naturally, we expected these release events to be Ca^{2+} sparks.

To our considerable surprise, this was not the case. As illustrated in figure 1B and 1C, Ca^{2+} release signals activated by flash-photolytic Ca^{2+} concentration jumps were always spatially homogeneous, irrespective of the power applied (14). Even reducing the power of the UV-flash to the threshold of CICR, to trigger only few RyRs did not result in circumscribed Ca^{2+} release events which could be resolved with the confocal microscope. Nevertheless, inhibiting CICR by applying ryanodine dramatically reduced the amplitude of the Ca^{2+} release signals and changed their kinetics (by only leaving the rapid photolytic Ca^{2+} concentration change). This represents pharmacological evidence that CICR was doubtlessly activated by the photolytical Ca^{2+} liberation. From the observation of SR Ca^{2+} release remaining spatially homogeneous under all conditions, we concluded that Ca^{2+} release occurred most likely through a Ca^{2+} signaling event that was too small to be resolved microscopically. Since the smallest SR Ca^{2+} release signal we could imagine was due to the brief opening of a single RyR (i.e. a Ca^{2+} quark), we proposed that a large number of such events were underlying the spatially homogeneous SR Ca^{2+} release signal we had observed.

5. SR Ca^{2+} RELEASE ACTIVATED BY TWO-PHOTON PHOTOLYSIS

This provocative conclusion was not immediately accepted by everyone working in the field. One consideration was that the hypothesis of a Ca^{2+} quark was essentially based on a negative result, or in other words, we had not optically resolved these events. Furthermore, the simultaneous use of UV-flash photolysis of caged Ca^{2+} compounds and laser-scanning confocal microscopy was a novel approach which could have its own intricacies and problems. In particular, the concern was expressed that the focal plane of the UV-flash might have been quite different from the focus of the laser beam exciting Fluo-3, even though the UV-flash was illuminating the entire cardiac myocyte. Indeed, we had previously determined the axial chromatic aberration of several microscope lenses used for confocal microscopy. The aberration was measured to be in the range of several µm, which is several-fold larger than the size of the point-spread function in the vertical direction (aberration was determined for confocal UV-excitation at wavelengths of 355 nm and 488 nm, respectively (18). Because of this chromatic aberration a UV-flash could, in principle, elicit Ca^{2+} sparks distant from the focal plane of the imaging system. Even a short distance would allow the Ca^{2+} sparks to diffuse together before reaching the plane of observation. Fortunately, a new photolytic technique bypassing the chromatic aberration problem inherent in UV-light became recently available. Instead of exciting a fluorescent molecule (or the chromophore of a caged compound) with a single UV photon, excitation can be achieved by near simultaneous absorption of several photons of longer wavelength (19). Instead of delivering UV-flashes at 355 nm to the entire cell, we created a stationary focus from a mode-locked Ti:sapphire laser running at a wave-length of 710 nm (20). The microscope objectives are well corrected for red light and therefore the axial chromatic aberration problem was minimal. In addition, because of the quadratic dependence of excitation on power, photorelease of Ca^{2+} only occurred in a volume corresponding to the diffraction limited focus of the red laser light. As shown in figure 2 this allowed us to create a point source of Ca^{2+} within a cardiac myocyte and ensured perfect parfocality between the two-photon excitation photolysis (TPP) and the plane imaged with the Ca^{2+} indicator Fluo-3. Using this photolytic technique, we were again not able to elicit Ca^{2+} sparks as all-or-none events. Despite the fact that the localized photolytic signals shared many spatial and temporal features with Ca^{2+} sparks (figure 2A), their amplitude was graded with the power of the photolytic laser over an extensive range of power levels. Only when the cells and Ca^{2+} stores were heavily loaded with Ca^{2+}, TPP could initiate regenerative Ca^{2+} release from the SR spreading along the cell as a triggered Ca^{2+} wave. When the power of the TPP laser was reduced down to the limit where photolytic signals could barely be resolved, we sometimes detected very small Ca^{2+} release events subsequent to the TPP trigger (Figure 2B). When comparing the Ca^{2+} signal mass of such small events with

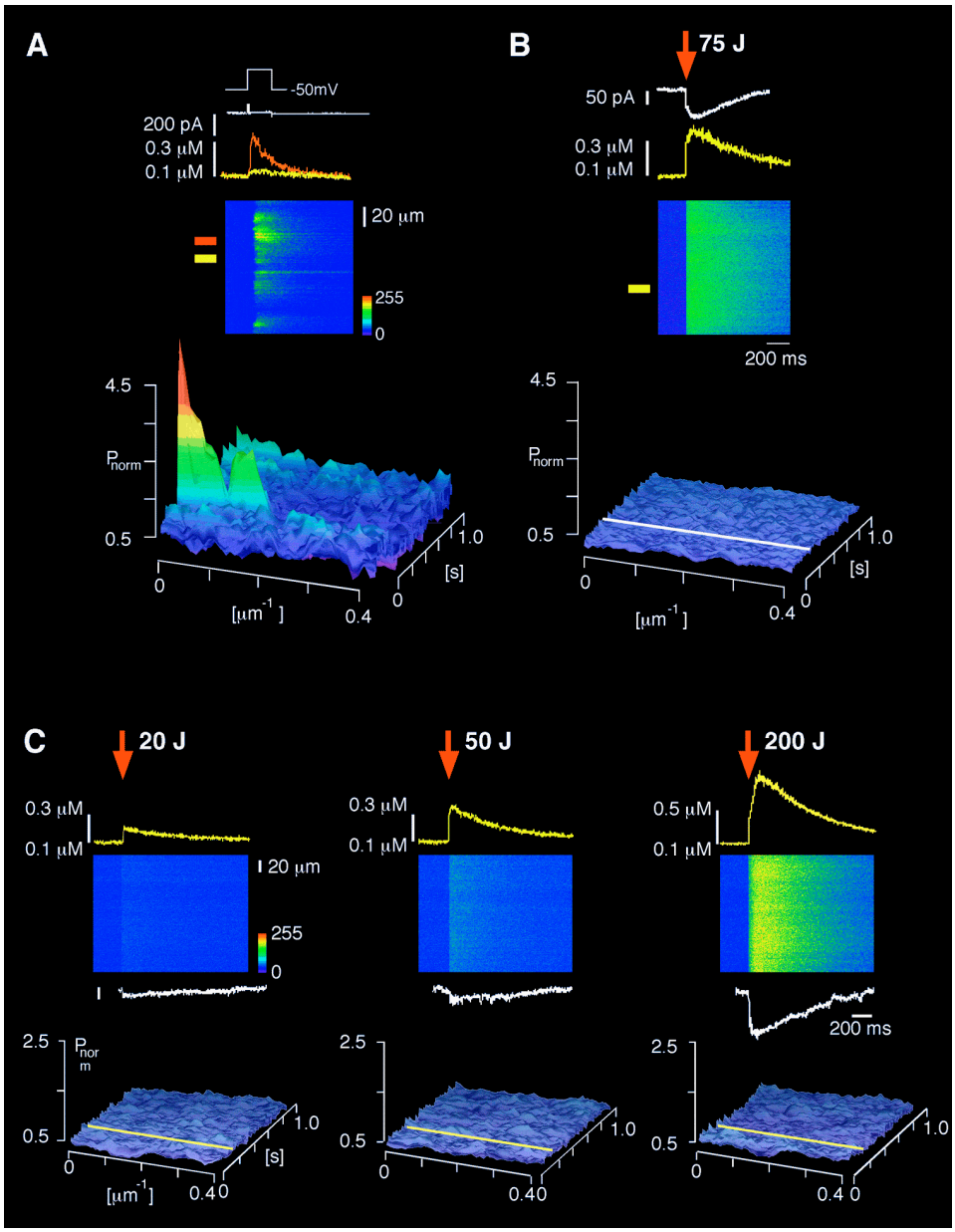

Figure 1. Comparison of Ca^{2+} transients induced by I_{Ca} and by homogeneous photochemical Ca^{2+} jumps in guinea-pig ventricular myocytes. **A**, a voltage-clamp depolarization from -50 mV to +5 mV activated a small $I_{Ca.L}$ due to a partial block by verapamil (10 µM). The line-scan image reveals several localized Ca^{2+} signals. These Ca^{2+} sparks rose to 350 nM (red trace) while the changes of $[Ca^{2+}]_i$ in regions between sparks were limited to about 25 nM (yellow trace). The power spectra in the spatial domain (computed with a fast Fourier transform, FFT, algorithm) exhibited a pronounced burst of low frequencies characteristic for localized Ca^{2+} signals. **B**, in the same cell a UV-flash was applied at a holding potential of –50 mV resulting in an inward current due to Ca^{2+} removal mediated by the Na-Ca exchange (I_{NaCa}). The Ca^{2+} transient (yellow) suggests a Ca^{2+} jump to 375 nM and the line-scan image reveals no spatial non-uniformities. This is confirmed in the flat power spectra (the white line indicates the moment of flash). The homogeneous photolytic Ca^{2+} trigger did not elicit Ca^{2+} sparks. **C**, UV-flashes of increasing energy were followed by I_{NaCa} and Ca^{2+} transients of increasing amplitude. The elicited Ca^{2+} signals were spatially uniform and the power spectra were flat at all energies (the yellow lines indicate the moment of the UV-flash). At a discharged energy of 20 J, the photolytically generated increase of $[Ca^{2+}]_i$ was completed within 2 ms, while a at 50 J a slower release component resulting from CICR followed the initial Ca^{2+} jump (photolytic released Ca^{2+}). This slow SR Ca^{2+} release component was even more pronounced when the flash energy was raised further to 200 J. Ca^{2+} sparks were not elicited by UV-flash photolysis of caged Ca^{2+} independent of the energy discharged. Homogeneous flash photolysis seemed to trigger Ca^{2+} release units that were substantially smaller in size, amplitude or duration than Ca^{2+} sparks. Scale bar: 20 pA (modified from 14).

Ca^{2+} sparks (derived by integrating the Ca^{2+} concentration over time and space occupied by a signal), it turned out that 20 - 40 times less Ca^{2+} was released for such a signal than for a single Ca^{2+} spark. Therefore, these small events might correspond to Ca^{2+} quarks, but we have no possibility to rule out the involvement of more than one RyR channel.

6. SR Ca^{2+} RELEASE ACTIVATED BY NA-CA EXCHANGE RUNNING IN THE Ca^{2+} INFLUX MODE

All observations of homogeneous Ca^{2+} release or small Ca^{2+} events described above depended, in one way or another, on photochemical techniques. However, several independent findings also suggest the genuineness of SR Ca^{2+} release units smaller than Ca^{2+} sparks in cardiac muscle. One example is detailed below. In cardiac muscle, the Na-Ca exchange is one of the most important pathways for removal of Ca^{2+} during relaxation (21). In the steady state, the Na-Ca exchanger thereby balances Ca^{2+} influx occurring via L-type Ca^{2+} channels from beat to beat (22). However, depending on the prevailing electrochemical gradients for Ca^{2+} and Na^{+}, the Na-Ca exchange can also run in a Ca^{2+} influx mode. This mode has been known for many years to underlie slow tonic contractions of cardiac muscle, for example after removal of extracellular Na^{+}. More recently, several laboratories found experimental conditions, under which Ca^{2+} influx via Na-Ca exchange was able to trigger Ca^{2+} release from the SR, notably after eliciting Na^{+} current by depolarizing the cell membrane (23). Using a laser-scanning confocal microscope and the fluorescent Ca^{2+} indicator Fluo-3, it was even possible to detect the small amount of Ca^{2+} which was entering the cell from the extracellular space (24). This tiny Ca^{2+} signal presumably corresponds to the trigger for CICR mediated by the Na-Ca exchange running in the Na^{+} removal (i.e. Ca^{2+} influx) mode. Initially, Na^{+} and Ca^{2+} enter the diadic cleft and since the diffusion of Na^{+} and Ca^{2+} in this narrow space is likely to be restricted, concentration changes for Na^{+} and Ca^{2+} in this space were predicted to be sufficiently large to initiate CICR (25). When we applied the same instrumentation and experimental approach to identify the nature of the elementary SR Ca^{2+} release events prevailing under these conditions, we observed homogeneous SR Ca^{2+} release signals reminiscent of those seen after UV-flash photolytic liberation of Ca^{2+}. Moreover, the Ca^{2+} signals were spatially homogeneous irrespective of the size of the depolarization and the Na^{+} current (26). Control experiments with pharmacological tools provided the necessary evidence to confirm that SR Ca^{2+} release had actually been triggered by Na-Ca exchange reverse mode. An interesting collateral observation provided a direct confirmation that the homogenous Ca^{2+} release occurred via a pathway distinct from the usual activation of Ca^{2+} sparks by L-type Ca^{2+} channels. Sometimes, Ca^{2+} sparks appeared to be superimposed on top of the homogeneous Ca^{2+} release signals. These triggered sparks were clustered during the first few milliseconds of the Ca^{2+} transient, i.e. exclusively during flow of I_{Na}. They could be abolished by inhibitors of L-type Ca^{2+} channels and their appearance could be boosted by increasing the series resistance of the patch-clamp electrodes. Based on these results we concluded that the superimposed Ca^{2+} sparks were triggered by the activation of a few L-type Ca^{2+} channels during voltage-escape which is inevitably introduced by large Na^{+} currents. Therefore, the strikingly different spatial features of the two types of Ca^{2+} release made it clear that the homogenous Ca^{2+} release was not simply the consequence of a large number of synchronized Ca^{2+} sparks. Instead, it appeared to be a different mode of Ca^{2+} release, possibly arising from the activation of a considerable number of Ca^{2+} quarks.

7. ELEMENTARY SR Ca^{2+} RELEASE SIGNALS IN SKELETAL MUSCLE

As mentioned above, several laboratories have identified Ca^{2+} sparks in skeletal muscle preparations (for example see (5-6). Skeletal muscle excitation-contraction coupling shares many similarities with cardiac Ca^{2+} signaling. However, despite the ultrastructural and molecular resemblance of the two cell types, there are remarkable differences that need to be considered before drawing conclusions from comparative studies. First of all, in skeletal muscle the RyR1 (and in some muscle types the RyR3) isoform is expressed, while cardiac muscle has the RyR2 (27). These isoforms show distinctly different behaviors for a variety of parameters, most notably for Ca^{2+} dependent activation and inactivation. Equally important, the initial steps of skeletal muscle EC-coupling are thought to rely on a direct mechanical coupling between the voltage-sensors and the RyRs. Afterwards, this voltage-induced Ca^{2+} release is also amplified by Ca^{2+}-induced Ca^{2+} release. In contrast, in cardiac muscle the earliest step of EC-coupling is Ca^{2+} influx via L-type Ca^{2+} channels, which is subsequently amplified by CICR (27). Thus, there are several reasons why one might expect to find more than one type of Ca^{2+} release in skeletal muscle. In one experimental study, Ca^{2+} signals were analyzed in frog skeletal muscle cells held in a double-vaseline gap voltage-clamp setup (28). Using the fluorescent Ca^{2+} indicator Fluo-3 and the line-scan mode of a confocal microscope, Ca^{2+} sparks were readily observed during near-threshold voltage-clamp depolarizations (to -58 mV). However, small depolarizations to -72 mV only sporadically elicited Ca^{2+} sparks. Nevertheless, a slight elevation of the Ca^{2+} concentration was consistently observed with every tiny depolarization. The authors proposed that this change of $[Ca^{2+}]_i$ must have resulted from Ca^{2+} signaling events smaller than Ca^{2+} sparks. This interpretation was further supported by a pharmacological experiment (see figure 3). Tetracaine is known to inhibit SR Ca^{2+} release via CICR (29). When voltage-clamp depolarizations to -58 mV were applied, the line-scan images revealed an initial synchronized surge of Ca^{2+} release, Ca^{2+} sparks, giving rise to a steady elevation of average $[Ca^{2+}]_i$. Superfusing the skeletal muscle cells with tetracaine eliminated the initial synchronized Ca^{2+} spike and the subsequent Ca^{2+} sparks. However, a small Ca^{2+} release signal again remained, probably originating directly from voltage-induced Ca^{2+} release. Taken together, it was proposed that in skeletal muscle voltage-induced Ca^{2+} release gives rise to “small

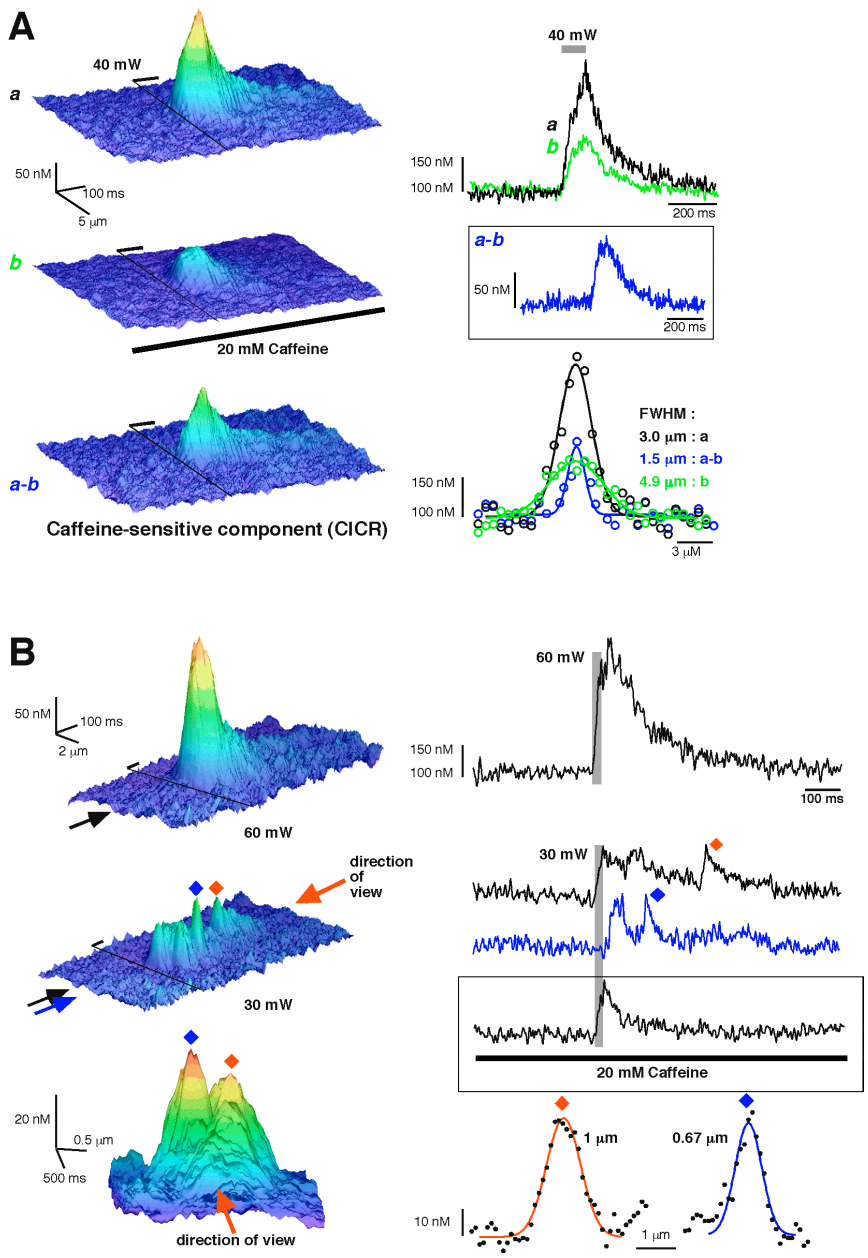

Figure 2. Ca^{2+} quark-like signals triggered by TPP in guinea-pig ventricular myocytes. **Aa** shows a Ca^{2+} signal generated by TPP at 40 mW (duration, 80 ms). Application of 20 mM caffeine reduced the photolytic Ca^{2+} transient significantly (***Ab***). Line tracings derived from the individual Ca^{2+} transients and the spatial spreading of the three Ca^{2+} release events are compared and illustrated in the right column. The caffeine-sensitive component arising from CICR is shown in **Aa-b** and in the inset (**a-b**). Interestingly, the event attributable to CICR was considerably smaller in amplitude than a typical Ca^{2+} spark. In addition to the smaller amplitude, the caffeine-sensitive difference signal also exhibited less spatial spreading (FWHM ˜ 1.5 μm) than a typical Ca^{2+} spark and than the TPP signal itself (FWHM ˜ 4.9 μm). The detection of a small caffeine-sensitive component indicates that TPP did trigger local CICR, possibly involving Ca^{2+} release events that are smaller than a Ca^{2+} spark, both in terms of amplitude and spatial spread. **B** shows a Ca^{2+} signal triggered by TPP at 60 mW (duration, 25 ms) and the corresponding time course of the Ca^{2+} signal, most likely containing a CICR component. Uncaging of DM-nitrophen slightly below threshold (30 mW) for SR Ca^{2+} release was followed by several tiny Ca^{2+} transients. A view from the end of the trace (in the direction of the red arrow) is depicted below to emphasize the spatial separation of the small Ca^{2+} release events. The time course of the tiny Ca^{2+} transients illustrated $[Ca^{2+}]_i$ at the location of photolysis (upper trace, red diamond) and ˜ 0·5 μm beneath this location (lower trace, blue diamond). The inset shows the TPP signal in the presence of 20 mM caffeine used for subtraction. The spatial characteristics of fundamental Ca^{2+} release signals are shown in more detail below. On average, the distance between the small Ca^{2+} signaling events triggered by TPP was ˜ 0.4 μm while the average amplitude of Ca^{2+} quarks was ˜ 37 nM (modified from 20).

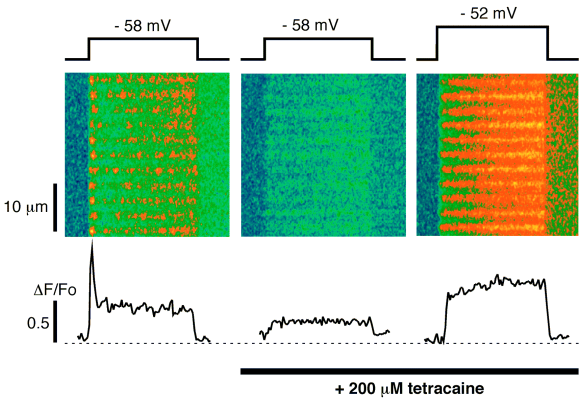

Figure 3. Tetracaine eliminates sparks, but spares "small event" Ca^{2+} release in frog skeletal muscle. Line scan images of normalized fluorescence in reference and in 200 μM tetracaine reveal triadic gradients of fluorescence, showing that tetracaine eliminated the peak of Ca^{2+} release and the occurrence of Ca^{2+} sparks. Fluorescence recorded at higher depolarization shows substantial Ca^{2+} release in tetracaine, but without peak or Ca^{2+} sparks. That tetracaine eliminated sparks at all voltages supports CICR as the activation mechanism, not just for some, but for all sparks (modified from 28).

event Ca^{2+} release" (presumably analogous to Ca^{2+} quarks) which subsequently triggers larger Ca^{2+} signals (i.e. Ca^{2+} sparks) by CICR.

8. Ca^{2+} RELEASE SIGNALS IN A HETEROLOGOUS EXPRESSION SYSTEM

Combining molecular biology techniques with Ca^{2+} imaging methods represents an almost ideal approach for engineering less complex experimental models. After the skeletal and cardiac muscle isoforms of the RyR (i.e. RyR1 and RyR2) had been cloned and sequenced (30-31), such an approach became possible and has been used to examine the elementary Ca^{2+} signaling events of RyRs heterologously expressed in Chinese hamster ovary (CHO) cells (see figure 4 and (32-33). In these cells, Ca^{2+} release could be elicited by applying puffs of caffeine, a compound which increases the open probability of the RyRs by rendering them Ca^{2+} sensitive to an extent, which allows even resting $[Ca^{2+}]_i$ to trigger Ca^{2+} release. In each of the two studies, caffeine-activated Ca^{2+} release from the Ca^{2+} stores was always spatially homogeneous and no Ca^{2+} sparks were resolved. Although the ultrastructural disposition of the RyRs expressed in these cells is not precisely known, it is obvious that major components of the microarchitecture of striated muscle cells are missing. Therefore, RyRs were certainly present in a much less organized distribution. Most likely, they were not aggregated in larger clusters of channels, since no dyads or triads were present and since the cytoskeletal elements for such a differentiated ultrastructure were missing. Therefore, the expressed RyRs may have been localized in the membrane of the endoplasmic reticulum of CHO cells in a quite isolated or at least less densely clustered fashion. The homogeneous Ca^{2+} release signals occurring via such spatially isolated channels then would be consistent with the view that the synchronized activation of more than one RyR is required to generate a detectable Ca^{2+} spark. Or, in other words, Ca^{2+} release in CHO cells expressing RyR1 or RyR2 may occur as (unresolved) Ca^{2+} quarks.

9. PERSPECTIVE

After many attempts to determine the number of RyRs contributing to a Ca^{2+} spark, everyone would probably agree that with an isolated technique, such as confocal microscopic imaging of Ca^{2+} signals, it will be very difficult to answer all questions related to the generation of a Ca^{2+} spark or to address the associated issues of channel activation and inactivation. What appears to be more promising are efforts to combine information obtained with complementary techniques, such as electrophysiology, Ca^{2+} imaging, ultrastructural studies, molecular biology and transgenic animal approaches as well as biochemical tools. A strategy which has been used in several studies, was to apply a model of Ca^{2+} diffusion and Ca^{2+} buffering in an attempt to correlate the Ca^{2+} spark amplitude (or signal mass) with single channel flux data, for example obtained from lipid bilayer experiments (1, 5, 34-35). When choosing such an approach, one has to keep in mind that the size of a local Ca^{2+} signal, such as a Ca^{2+} spark or Ca^{2+} quark, is ultimately determined by many factors. These include the diffusion of Ca^{2+} inside the cell, the Ca^{2+} buffer capacity of the cell and the Ca^{2+} binding kinetics and mobility of the Ca^{2+} indicator. Equally important, several properties of the Ca^{2+} release channels themselves directly affect the resulting Ca^{2+} signals, such as the amount of Ca^{2+} flux carried by an open channel and the gating behavior of the channel protein. Gating and Ca^{2+} flux of membrane channels can be conveniently analyzed using electrophysiological techniques. As already mentioned, a lipid bilayer technique has been developed which allowed studies of channel conductance and gating with isolated RyRs, as well as pharmacological tests. While this technique is generally used to characterize the channel under steady-state conditions (for reviews see 36-37), some transient state studies have already been carried out by combining the lipid-bilayer with flash-photolysis techniques or with rapid solution changes (38-40). Such experiments more closely correspond to the physiological situation where the RyRs are only activated transiently. In experiments designed to mimic intracellular solutions as closely as possible, the previously established estimate for the Ca^{2+} current carried by a single cardiac RyR turned out to be too large and has been lowered to less than 0.6 pA (12). Although this current was still recorded under the highly artificial conditions of a lipid bilayer experiment, it represents the "best" data currently available. However, when substituting this current in numerical simulations of Ca^{2+} sparks, one would still need to make several assumptions regarding the Ca^{2+} diffusion in the cell and in the diadic cleft, but also assume a Ca^{2+} buffer capacity of the cell.

A preferable experiment would be to record the Ca^{2+} current flowing through a Ca^{2+} channel directly while simultaneously measuring the resulting Ca^{2+} signal. Such a

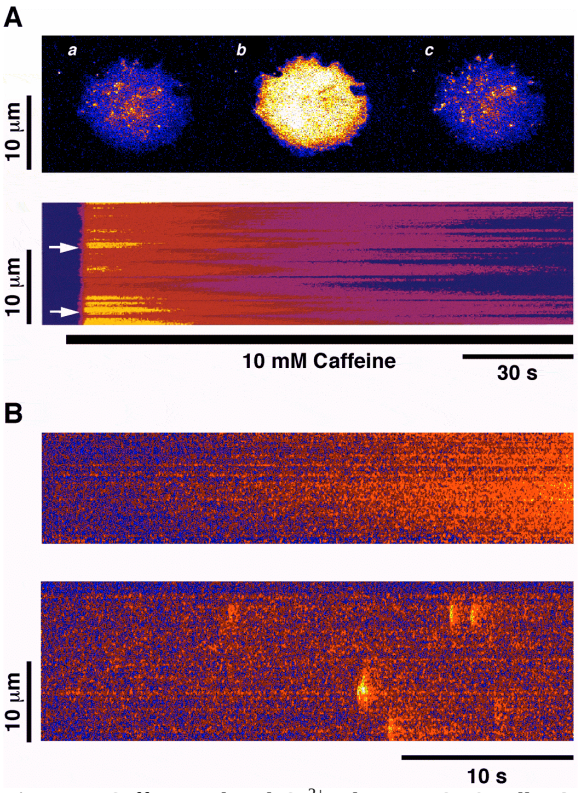

Figure 4. Caffeine-induced Ca^{2+} release in CHO cells. **A**, Spatial and temporal patterns of caffeine-induced Ca^{2+} release in CHO cells expressing the full length skeletal muscle RyR. Images of Fluo-3 fluorescence in CHO cells expressing the full length RyR: (**a**) control, (**b**) 0.5 s after addition of 10 mM caffeine, and (***c***) 5 min after washout of the caffeine. The lower panel shows a compressed line-scan image of Fluo-3 fluorescence recorded from a single CHO cell exposed to 10 mM caffeine. The distribution of $[Ca^{2+}]_i$ in response to caffeine was inhomogeneous. There appeared to be 'hot regions' where the level of $[Ca^{2+}]_i$ was higher than in other places, as indicated by the arrows. These hot spots may correspond to regions where the density of Ca^{2+} release channels was high. **B**, Lack of Ca^{2+} sparks in CHO cells expressing RyR. Line-scan image of fluorescence ratio (Fluo-3) in CHO cells during the initial rise in $[Ca^{2+}]_i$ in the presence of 0.5 mM caffeine. $[Ca^{2+}]_i$ rises very slowly from resting levels, but no sparks are evident. In contrast, typical spontaneous Ca^{2+} sparks were seen in a cardiac myocyte (lower panel). Both line-scan images were obtained and presented at exactly the same spatial and temporal resolution, with the same confocal microscope (modified from 32).

combined set of data would allow a direct correlation of Ca^{2+} flux and Ca^{2+} signal, without having to rely on model dependent and poorly known assumptions. In fact, such an experimental study has recently been published (41). The strategy for this ingenious and challenging experiment was to record Ca^{2+} current via a single cardiac L-type Ca^{2+} channel in a cell-attached configuration of the patch-clamp technique, while at the same time imaging the resulting Ca^{2+} sparks, or, after inhibition of the SR, record the Ca^{2+} signals arising from the Ca^{2+} influx via a single L-type Ca^{2+} current (termed a "Ca^{2+} sparklet"). By comparing the L-type Ca^{2+} current and the corresponding Ca^{2+} sparklet, the amount of Ca^{2+} flux required to generate a Ca^{2+} spark could be estimated directly. The authors concluded that for a typical Ca^{2+} spark the activation of about 4-6 RyRs was required. Thus, this finding also suggests that from within a dyad with its ultrastructural cluster of about hundred RyRs (13), only a small subset is actually activated. The question why only such a small fraction of the RyRs within a cluster are activated, remains open. Here, we obviously have the same conceptual issue that needs to be addressed in order to explain, why Ca^{2+} quarks can exist without immediately triggering a full-blown Ca^{2+} spark (by activating all neighboring channels).

While many readers may consider the precise number of Ca^{2+} channels contributing to a Ca^{2+} spark a hair-splitting detail, most would certainly agree that we need to understand the activation, inactivation and regulation of RyRs from the level of a single channel, through the somehow coordinated behavior of channel clusters (42-43), up to the complexity of intact cells and cardiac muscle. In order to have a complete picture it is essential to understand all mechanisms by which the channels grouped within a dyad can communicate with each other. We are convinced that future experiments using interdisciplinary approaches will provide the information necessary to solve this puzzle.

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Send correspondence to: Dr Ernst Niggli, Department of Physiology, University of Bern, Bühlplatz 5, 3012 Bern, Switzerland, Tel: +41 31 631 8730, Fax: +41 31 631 4611, E-mail: niggli@pyl.unibe.ch, URL: http://beam.to/calcium_quark