Ca\textsuperscript{2+} Sparks in Rabbit Ventricular Myocytes Evoked by Action Potentials
Involvement of Clusters of L-Type Ca\textsuperscript{2+} Channels

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Abstract—It is not clear how many L-type Ca\textsuperscript{2+} channels (LCCs) are required to ensure that a Ca\textsuperscript{2+} spark is triggered during a normal mammalian action potential (AP). We investigated this in rabbit ventricular myocytes by examining both the properties of sparks evoked by APs and the activity of LCCs. We measured Ca\textsuperscript{2+} sparks evoked by repeated APs with pipettes containing 2 mmol/L EGTA and single LCC activity in cell-attached patches depolarized to +50 mV using pipettes containing 110 mmol/L Ba\textsuperscript{2+}. With 2 mmol/L Ca\textsuperscript{2+} in the external solution, we observed sparks at the beginning of every evoked AP at numerous locations. Each spark was observed repeatedly at a fixed location and began during a limited interval after the AP peak. These sparks occurred with a probability of approximately unity. However, the chance that an LCC does not open during the interval when a spark is triggered is quite high (=0.13). Therefore, because single channels open with a probability significantly lower than 1, more than one LCC must be available to ensure that sparks are triggered with a probability of approximately unity. We conclude that it is likely that a cluster of LCCs is involved in gating a cluster of ryanodine receptors at the beginning of an AP. (Circ Res. 2003;92:532-538.)

Key Words: Ca\textsuperscript{2+} channels ■ Ca\textsuperscript{2+} sparks ■ excitation-contraction coupling ■ Ca\textsuperscript{2+} triggers ■ trigger clusters

Calcium sparks are local Ca\textsuperscript{2+} release events from the sarcoplasmic reticulum (SR) and are believed to be the elementary basis of the Ca\textsuperscript{2+} transient. Thus, summation of many of these sparks is thought to underlie the Ca\textsuperscript{2+} transient. Cheng et al\textsuperscript{1} originally proposed that a spark represented the flux of Ca\textsuperscript{2+} through a single SR release channel or ryanodine receptor (RyR). This idea has been discarded in favor of the idea that a spark originates from a cluster of 10 to 40 RyRs,\textsuperscript{2-6} although some anatomical results suggested that as many as 200 RyRs may form a cluster.\textsuperscript{7} Separation of clusters of RyRs offers a partial explanation for local control of excitation-contraction coupling.\textsuperscript{8}

A question of central importance is how L-type Ca\textsuperscript{2+} currents trigger sparks. In particular, how many L-type Ca\textsuperscript{2+} channels (LCCs) are required to ensure a given probability of spark production? There does seem to be fairly broad agreement that a single LCC opening can trigger a spark.\textsuperscript{9-14} These conclusions on triggering sparks are based on experimental conditions where the probability of sparks was intentionally reduced by using Ca\textsuperscript{2+} antagonists or low [Ca\textsuperscript{2+}], or by applying negative potentials. Under these conditions, it might be reasonable to conclude that a single LCC can gate RyRs with low probability. Few studies, however, have been conducted with unblocked L-type Ca\textsuperscript{2+} current, i.e., with more physiological [Ca\textsuperscript{2+}], or in the absence of Ca\textsuperscript{2+} antagonists. Under these conditions, sparks may be expected to appear more frequently but must still be triggered by Ca\textsuperscript{2+} flux that enters through LCCs close to clusters of RyRs. In this study, we investigated the properties of sparks and the activity of LCCs in rabbit ventricular myocytes under the conditions where spark probability and L-type Ca\textsuperscript{2+} current was not reduced.

Materials and Methods

Animals
We used adult New Zealand White rabbits (2.0 to 3.0 kg; Charles River Labs, Wilmington, Mass) housed according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health), completely anesthetized with intravenous administration of sodium pentobarbital (50 mg/mL). Myocytes were isolated enzymatically.\textsuperscript{15}

Confocal Imaging
We used fluo-3AM (Molecular Probes) and a BioRad MRC-1024 laser-scanning confocal microscope system.\textsuperscript{13} Myocytes were placed with their long axis within ±10 degrees along the longitudinal axis of the imaging window. All images were acquired in linescan mode with 0.15 μm and 2 ms per pixel resolution. As the confocal system could not perfectly synchronize images with the external trigger, we also imaged stimuli simultaneously to align line-scan images. The whole-cell patch-clamp technique was applied to myocytes to stimulate and record APs. We recorded APs elicited with 0.2 nA m and 2 ms per pixel resolution. As the confocal system could not perfectly synchronize images with the external trigger, we also imaged stimuli simultaneously to align line-scan images. The whole-cell patch-clamp technique was applied to myocytes to stimulate and record APs. We recorded APs elicited with 0.2 nA m and 2 ms per pixel resolution.
with NaOH. Pipettes (2.0 to 2.5 MΩ) were filled with a solution containing (in mmol/L) KCl 110, K-ATP 5, MgCl, 5, EGTA 2, CaCl, 0.54, NaCl 10, and HEPES 20. The pH was adjusted to 7.1 with KOH. We used an internet-based calculating program MAXCH-ELATOR (available at http://www.stanford.edu/~cppton/max-ch.html) to calculate free [Ca\textsuperscript{2+}] in the pipette solution (~90 mmol/L at room temperature). All experiments were performed at room temperature. Data were filtered at 1 kHz and acquired with a Digidata 1200 acquisition system and pClamp8 software.

We analyzed the fluorescence images with the public domain NIH Image program (available at http://rsb.info.nih.gov/nih-image/).

**Unitary Current Recording**

We recorded single LCC currents in cell-attached patches. Pipettes (2.5 to 4.0 MΩ) coated with Sylgard 184 (Dow Corning) were filled with the pipette solution containing (in mmol/L) BaCl\textsubscript{2} 110, HEPES 10, and TEACl 5. The pH was adjusted to 7.4 with TEAOH. The resting potential was set to zero with high-K\textsuperscript{+} bath solution containing (in mmol/L) KCl 140, dextrose 11, EGTA 2, and HEPES 24. The pH was adjusted to 7.4 with KOH. Unitary currents were recorded with patches of >30 GΩ seal resistance using an Axopatch 200A patch clamp. We applied voltage commands from a holding potential (in mmol/L) KCl 110, K\textsubscript{2} ATP 5, MgCl\textsubscript{2} 5, EGTA 2, CaCl\textsubscript{2} 90 nmol/L, and HEPES 20. The pH was adjusted to 7.1 with KOH. We used an internet-based calculating program MAXCH-ELATOR (available at http://www.stanford.edu/~cppton/max-ch.html) to calculate free [Ca\textsuperscript{2+}] in the pipette solution (~90 mmol/L at room temperature). All experiments were performed at room temperature. Data were filtered at 1 kHz and acquired with a Digidata 1200 acquisition system and pClamp8 software. All the experiments were performed at room temperature.

We subtracted baseline current (uncompensated capacitance current) from each sweep before analysis. The variation of capacitance current within 2 ms after depolarization was so large that we did not calculate nor analyze this 2-ms period. To calculate current amplitudes and open probabilities, we used all-point histograms and Gaussian fitting methods.\textsuperscript{16} We detected the probabilities of null events by using a 50% threshold.\textsuperscript{16}

**Results**

**Ca\textsuperscript{2+} Sparks in Rabbit Ventricular Myocytes**

Evoked sparks are usually measured with procedures that reduce the probability of evoking sparks, including application of Ca\textsuperscript{2+} antagonists\textsuperscript{1,10} or low [Ca\textsuperscript{2+}].\textsuperscript{2,9} However, without these procedures it is difficult to detect sparks because they are numerous and fused (Figure 1A, bottom). Without these procedures it is difficult to detect sparks because they are numerous and fused (Figure 1A, bottom). Others measured Ca\textsuperscript{2+} release events in rat myocytes by dialyzing them with EGTA-containing solution.\textsuperscript{17} These authors presented arguments that EGTA increases the chance of detecting sparks by preventing their diffusion and improving signal-to-noise values by reducing background fluorescence. Without EGTA in the pipette solution (Figure 1A, bottom), some local regions of high fluorescence intensity appeared at the beginning of the AP, but eventually formed a fused Ca\textsuperscript{2+} transient (Figure 1B). Myocytes contracted during this elevation of Ca\textsuperscript{2+}. With 2 mmol/L EGTA in the pipette solution (Figure 1C, middle), there were several regions of high fluorescence intensity at the beginning of the AP. By self-ratioing this fluorescence image, we were able to resolve sparks (Figure 1C, bottom). These spark appearances are limited to the beginning of APs. They disappeared after 10-minute treatments with 1 mmol/L thapsigargin in the bath solution (data not shown), consistent with the idea that they are local release events through RyRs.

**At a Fixed Location, Sparks Occur During Every AP**

We investigated spark probability at fixed locations.\textsuperscript{2} With repeated stimuli, we obtained 50 self-ratioed images within 40 ms after each stimulus (Figure 2A). All sparks occurred only at the beginning of the repeated APs. The amplitudes of sparks were variable among locations. This can be explained if the centers of all sparks in the entire image were not located in the same confocal plane. There were some areas (eg, between locations a and b in Figure 2A) where sparks were not detected, suggesting the absence of T-tubules or SR junctions in these areas. Sparks could be signal-averaged (Figure 2B) because they occurred at almost the same time during each AP. Some sparks were fused (region g) and could not be separated in the averaged image. However, their amplitude profiles exhibit clearly discernable peaks. The distances between adjacent peaks are 1.65 μm (mean, range 1.35 to 2.1 μm) and are similar to the distances between T-tubules (1.8 μm).\textsuperscript{18} Spark profiles at various locations (Figure 2C) shows sparks always appeared after every stimulus with only one exception (see figure legends), hence the probability of Ca\textsuperscript{2+} spark occurrence evoked by APs is, at locations where they appear, approximately unity in rabbits. At a given location, the peak amplitudes of these sparks are similar. We examined 9 additional myocytes (25 to 50 consecutive stimuli), and obtained a series of less resolved, less bright sparks in each image. Nevertheless, these sparks also appeared with probability of approximately unity and their properties were similar to those in the representative myocyte.

**Properties of Sparks at a Fixed Site**

We examined the properties of a single spark by averaging F/F\textsubscript{0} images at the same location (Figure 3, bottom). The
The spark size was 1.8 μm (FWHM; full width at half maximum) in diameter and was similar to those reported in other species, e.g., 2.0 and 1.8 μm (FWHM) in rats and mice, respectively. The profile of the averaged spark center (Figure 3, middle) reveals some delay from stimulation to spark appearance. By examining all the 50 images, the activation (at 10% maximum) of the spark occurred within the limited interval (3 to 4 pixels or 6 to 8 ms) after the stimulation signal recorded simultaneously. There was less than 1 pixel (0 to 2 ms) of delay between applying a stimulus and scanning a stimulus signal at a given site. As the AP peak consistently occurred 4 ms after the stimulus signal (Figure 3, top), the latency from the AP peak to the activation of the spark was estimated to lie within a limited range from 2 to 6 ms.

**Unitary Ba$^{2+}$ Currents**

To investigate voltage-gated LCCs, we measured unitary Ba$^{2+}$ currents through LCCs by voltage clamp. The advantage of using Ba$^{2+}$ instead of Ca$^{2+}$ has been discussed extensively. Although many experiments on LCC behavior are conducted near 0 mV, we needed to investigate their behavior at more positive potentials such as +50 mV that correspond to the AP peak. Despite a poor signal-to-noise ratio at very positive potentials, we could analyze 3 sets of data from +10 to +50 mV. The current amplitudes at +10 and +50 mV are $-0.93 \pm 0.03$ and $-0.21 \pm 0.03$ pA (mean±SEM, n=3), respectively. The average channel conductance from +10 to +30 mV (we excluded +40 and +50 mV because the current-voltage relationship is not linear near its reversal potential) was $23 \pm 1$ pS (mean±SEM, n=3). Our value is consistent with other reports.

As the whole-cell current is the summation of many unitary currents, the ensemble averages are proportional to the whole-cell currents. Thus, they should reveal the voltage dependence of whole-cell currents through LCCs. At +10 mV, the current develops slowly and saturates after 20 ms (Figure 4A). At +50 mV, the current saturates within 5 ms (Figure 4B). The difference in time courses and amplitudes at
different voltages reveals the voltage dependence of the current activation.\textsuperscript{22} We conducted these measurements at a relatively high clamp-pulse frequency (10 Hz). The channel always closed less than 10 ms after the voltage command, consequently there were more than 70 ms of rest at −80 mV before each clamp pulse. However, there still may have been frequency dependent effects on the activation of the LCCs. We applied voltage commands from −80 mV to +10 mV to the same myocyte with the same frequency (0.33 Hz) as in the spark experiments. There was no detectable difference in time course of the ensemble average currents between high-frequency protocol (20 ms, 10 Hz in Figure 4A) and low frequency protocol (200 ms, 0.3 Hz in Figure 4C). Thus, we conclude there was no frequency-dependent effect when we measured unitary currents with this high-frequency protocol. The ensemble averages of our unitary Ba\textsuperscript{2+} currents show very slow or no decay after their peak. Cavalí et al\textsuperscript{23} showed slow decay in the ensemble average at 32° to 35°C. It is possible that lower temperature (22° to 24°C) in our experimental condition makes the decay even slower.

**Ca\textsuperscript{2+} Channel Activity Within 2 to 6 ms After the Beginning of Depolarization**

Because the sparks were activated within 2 to 6 ms after the peak of each AP, we investigated the behavior of LCCs during this period. The open probability of LCCs becomes greater when the voltage becomes more positive.\textsuperscript{22,24} However, there were some null sweeps not only at +10 mV (eg, sweeps No. 11 and 371 in Figure 5A) but also at +50 mV (eg, sweep No. 292 in Figure 5A). These were null for the entire duration of the sweeps except for the first 2 ms when we could not resolve nulls. If we limit the time window within 2 to 6 ms after depolarization, there were additional partially null sweeps (eg, sweeps No. 12, 151, and 389 in Figure 5A, and sweeps No. 102, 222, and 420 in Figure 5B).

Next, we calculated average probabilities of opening (P\textsubscript{open}) and the probabilities of null events (P\textsubscript{null}) within 2 to 6 ms after depolarizations to respective potentials (Figure 6). P\textsubscript{open} shows sigmoid voltage dependence and reaches its maximum \( \approx 0.67 \) near +50 mV. P\textsubscript{null} also shows inverse-sigmoid voltage dependence and reaches its minimum \( \approx 0.13 \) near +50 mV. Others have reported these sigmoid and inverse-sigmoid relationships of P\textsubscript{open}\textsuperscript{25} and P\textsubscript{null}\textsuperscript{24} versus voltage. P\textsubscript{null} at +50 mV (\( \approx 0.13 \)) indicates that the chance that a single LCC opens at least once within 2 to 6 ms after depolarization to +50 mV is \( \approx 0.87 \). This probability is much lower than the spark probability that is approximately unity.

**Discussion**

We infer that in rabbits, more than one LCC is required to gate RyRs and hence trigger sparks at high probability. It is essential to understand that this does not mean that a single LCC cannot trigger a cluster of RyRs. The reason for our conclusion is straightforward. Sparks evoked by APs appear during a short interval at the beginning of the AP with a probability of approximately unity (with a very few spark...
failures). However, depolarization to +50 mV (approximate peak AP voltage) does not always produce single LCC openings (they fail to open with a probability \( \approx 0.13 \)). A large body of evidence suggests that sparks are triggered by Ca\(^{2+}\) entry through LCCs. Thus, to ensure 100% gating of one or more RyRs, more than one LCC must be available to produce this gating frequency. However, different members of a cluster of LCCs could be involved on successive occasions.

The relationship between LCC openings and spark appearance was recently investigated by recording unitary Ca\(^{2+}\) currents with single-channel loose patches and the Ca\(^{2+}\) sparks produced by these currents.\(^{14}\) However, we could not use this direct approach to study the relationship between sparks and Ca\(^{2+}\) triggers. Instead, we used a less direct approach in which we compared the null probability of LCCs and the probability of sparks from separate experiments. We adopted this approach for the following reasons. First, we investigated spark probability at positive potentials during APs. The unitary current amplitude at +50 mV is so small (\(-0.21\) pA with 110 mmol/L Ba\(^{2+}\)) that we could not expect to record it with a loose patch. Second, it is possible that the structure of SR junction is impaired even with loose patches. Third, the direct approach required the use of FPL64176 to prolong LCC openings. Use of such agonists to prolong LCC openings seems necessary to detect unitary LCC currents and their concomitant sparklets. Thus, the coupling fidelity between LCC openings and sparks from their results may be inflated. In the following discussion, we assume every LCC opening can trigger a spark. This may overestimate the coupling fidelity but does not alter our main inference. We also discuss the limitations of our LCC measurements and show that these limitations do not alter our conclusion.

**How Many LCCs Are Involved in Triggering a Spark?**

With the probability of sparks (\(P_{\text{spark}}\)), the null probability of a single LCC (\(P_{\text{null}}\)), and the number (\(N\)) of channels in a cluster to trigger RyRs, we can obtain the following relationships. If we assume that every channel opening can trigger a spark, the chance of obtaining a spark (\(P_{\text{spark}}\)) is equal to the chance of obtaining at least one opening from any of the channels, ie, \(1-(P_{\text{null}})^N\). From our results \(P_{\text{null}}\) is \(\approx 0.13\). Thus, setting \(N=2\) makes \(P_{\text{spark}}=1-(0.13)^2=0.98\). Because \(P_{\text{spark}}\) is higher than this, we require more than 2 LCCs to be involved in triggering.

We have used Ba\(^{2+}\) as a charge carrier and from data obtained with this ion, we have attempted to make inferences about the behavior of an LCC when Ca\(^{2+}\) is the charge carrier. If we suppose that the first opening of a Ca\(^{2+}\) channel is responsible for gating sparks,\(^{26}\) inferences made with Ba\(^{2+}\) as a charge carrier could be valid. This is because we assume the first opening to depend only on voltage. Even though it is widely accepted that Ca\(^{2+}\) but not Ba\(^{2+}\) has the capacity to inactivate the channel when it passes through it,\(^{27}\) there is no a priori reason to assume that Ca\(^{2+}\) will affect \(P_{\text{null}}\) because this inactivation effect of Ca\(^{2+}\) cannot be expected to affect the first opening unless it is accumulated from beat to beat. If this were the case, our main conclusion would be strengthened.

Although we assume that every channel opening can trigger a spark, this may not be the case. The unitary current amplitude was \(-0.21\) pA at +50 mV with 110 mmol/L Ba\(^{2+}\) in pipettes. However, if we used 2 mmol/L Ca\(^{2+}\) in pipettes instead, the unitary current amplitude would be much smaller at +50 mV. By applying the Goldman-Hodgkin-Katz current...
equation\textsuperscript{21} with a known permeability ratio $P_{Na}/P_{Ca}$,\textsuperscript{28} we can calculate its amplitude at $+50$ mV with $2$ mmol/L $Ca^{2+}$ in pipettes to be only $-9.5$ fA. In our calculation, we assume that any short opening of LCC could trigger a spark.\textsuperscript{29} However, Viatchenko-Karpinski et al\textsuperscript{40} recently argued that very brief openings with such small current amplitudes might not trigger sparks at all. If this is true, the probability that an LCC does not gate any RyRs is greater than $P_{null}$ and hence $N$ must be 3 or more.

By recording through a 1-KHz filter, we missed single-channel events $<0.18$ ms in duration. Open time analysis\textsuperscript{18} at $+50$ mV revealed that missed open events were 5\% of the total open events. This would reduce actual $P_{null}$ to 0.11 but does not alter our conclusion of LCC clustering, particularly if these brief openings do not exhibit a high probability of triggering.\textsuperscript{40}

The clustering of LCCs is also supported by a number of studies. To produce a spark, approximately 10 to 40 RyRs are reported to be involved.\textsuperscript{2,5,31-33} The ratio of RyRs to dihydropyridine receptors was reported to be 3.7 in rabbit ventricular myocytes.\textsuperscript{34} If we assume RyRs and LCCs are mostly located at the SR junctions,\textsuperscript{35} 3 to 11 LCCs seems to be involved in gating RyRs. With our $P_{null}$ ($\sim0.13$), the values of $N$ from 3 to 11 produce values of $P_{null}$ very close to unity [eg, when $N=3, P_{null}=1−(0.13)^3=0.997$].

Zhou et al\textsuperscript{36} suggested far greater than 1:1 coupling fidelity between LCCs and RyRs under normal conditions. Initially, at least one RyR should be activated to produce a spark so that their results suggest the involvement of multiple LCCs in spark triggering. Takagishi et al\textsuperscript{37} and Harms et al\textsuperscript{38} reported that LCCs exist in large clusters in rabbit ventricular myocytes and live HEK293 cells, respectively. Our inference of clustering is consistent with these anatomical observations.

To maintain SR content, we resorted to evoking sparks with APs in the presence of a $Na^{+}$ gradient. Under these conditions, reverse-mode $Na^{+}$-$Ca^{2+}$ exchange may contribute to triggering SR release.\textsuperscript{39} On the other hand, Sipido et al\textsuperscript{40} have reported that reverse-mode $Na^{+}$-$Ca^{2+}$ exchange triggers SR $Ca^{2+}$ release but with a significant delay. Moreover, $Na^{+}$-$Ca^{2+}$ exchange is not very active at room temperature. However, there is evidence that L-type $Ca^{2+}$ current and reverse-mode $Na^{+}$-$Ca^{2+}$ exchange act synergistically.\textsuperscript{30,41} $Na^{+}$-$Ca^{2+}$ exchange is regulated by intracellular $Ca^{2+}$ at a high-affinity binding site.\textsuperscript{32} $Ca^{2+}$ influx through the exchanger is increased by that through LCCs.\textsuperscript{43,44} Therefore, when a brief opening of an LCC cannot produce sufficient accumulation of $Ca^{2+}$ to trigger sparks specially at positive potentials (eg, APs), it is possible that $Ca^{2+}$ entry through LCCs activates $Ca^{2+}$ entry by $Na^{+}$-$Ca^{2+}$ exchanger and these trigger a spark in concert when neither of them can do so alone. However, $Ca^{2+}$ influx through LCCs is still essential to trigger sparks. Thus, the possible involvement of $Na^{+}$-$Ca^{2+}$ exchanger in the triggering process does not alter our inference of LCC clustering.

**Importance of LCC Clustering for Excitation-Contraction Coupling**

Two aspects of RyR gating by clusters of LCC may be important in both normal and diseased hearts. In the normal hearts, these are gating RyRs at the beginning of APs and also gating RyRs with a probability of unity. The former will result in sparks occurring at the beginning of APs at numerous locations. This would favor homogenous myocyte contraction. The latter will result in sparks occurring with a probability of unity at those locations where they are activated. This would optimize trigger efficiency and account for our observations. In the failing heart, $Ca^{2+}$ sparks were observed with temporal and spatial heterogeneities.\textsuperscript{45} Sipido\textsuperscript{46} suggested that LCC clustering may explain this. If the number of available LCCs in a cluster decreases in the failing heart, the chance that a cluster gates RyRs will decrease and this will result in greater temporal dispersion of sparks, and sparks will not be uniformly triggered at the beginning of APs. Hyperactive LCCs in failing hearts\textsuperscript{47} may to some extent compensate for defective $Ca^{2+}$ triggers, but cannot completely prevent trigger failure.

A potential difficulty with our finding is that sparks occur with a probability of approximately unity. If all SR release units are activated, local control will not be responsible for increasing release. On the other hand, we do not know whether all SR release units are activated. Our results clearly showed that there are some T-tubular locations where sparks are not detected. It is possible that the presence of EGTA limits the activation of RyRs. This issue requires further investigation.

**Limitations**

Our experiments are performed at room temperature. At 37°C, LCCs will be more active.\textsuperscript{22,23} Even if these conditions reduce $P_{null}$ we still require LCC clustering to explain our results at room temperature. With the cell-attached patch-clamp technique, we recorded from LCCs on the surface membrane but not in the T-tubules. However, there is no priori reason to think that LCC activity on the surface membrane is different from that in T-tubules. The properties of rabbit sparks are similar to those observed in other species.\textsuperscript{2,19} Our measurements of LCC activity are also similar to those reported with other species.\textsuperscript{24} Studies that are consistent with LCC clustering\textsuperscript{2,5,31-38} have been performed in a variety of species. Thus, it seems reasonable to suggest that LCC clustering is not limited to the rabbit.

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**References**


