Ca^{2+} sparks evoked by depolarization of rat ventricular myocytes involve multiple release sites[1]

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ABSTRACT

AIM: To investigate the fundamental nature of calcium release events (Ca^{2+} 'sparks') evoked in rat ventricular myocytes during excitation-contraction (E-C) coupling. **METHODS:** High-resolution line-scan confocal imaging with the fluorescent calcium indicator and patch-clamp techniques were used to study the spontaneous Ca^{2+} sparks and sparks evoked by depolarization. **RESULTS:** 1) Line scans oriented along the length of the cell showed that both spontaneous sparks and sparks evoked by depolarization to -35 mV appeared to arise at single sites spacing about 1.8 μm apart (ie, the sarcomere length), and measurements of their longitudinal spread (full-width at half-maximal amplitude: FWHM) followed single Gaussian distributions with means of 2.6 μm. 2) Different to this, transverse line scans often revealed spontaneous and evoked sparks that appeared to arise near-synchronously from paired sites. Measurements of transverse FWHM of both spontaneous and evoked sparks showed bimodal distributions, which were fit well by the sums of two Gaussian curves with means of 1.8 and 2.9 μm for spontaneous sparks and with means of 1.9 and 3.1 μm for evoked sparks. Relative areas under the two Gaussian curves were 1.73:1 and 1.85:1, respectively, for spontaneous and evoked sparks. **CONCLUSIONS:** Ca^{2+} sparks evoked by depolarization are not 'unitary' events, but often involve multiple sites of origin along Z-lines, as previously shown for spontaneous sparks. Thus, Ca^{2+} released during sparks directly triggered by influx through L-type Ca^{2+} channels may, in turn, trigger neighboring sites. The restricted involvement of only a few transverse release sites preserves the essential feature of the 'local control' theory of E-C coupling.

INTRODUCTION

Ca^{2+} 'sparks' (localized releases of Ca^{2+} from the sarcoplasmic reticulum) occur spontaneously in mammalian heart muscle[1] and are evoked during excitation-contraction (E-C) coupling through a process of Ca^{2+}-induced Ca^{2+} release (CICR) by the local Ca^{2+} gradients existing under open L-type Ca^{2+} channels[2-7]. The 'local control' theory of E-C coupling holds that Ca^{2+} sparks are recruited, independently of one another, during L-type Ca^{2+} currents to produce the whole-cell Ca^{2+} transient[3-6]. Despite the inherently regenerative nature of CICR, the graded relationship between whole cell

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Ca^{2+} release from the sarcoplasmic reticulum and whole-cell Ca^{2+} current[8-10] can then be explained on the basis that Ca^{2+} release is evoked exclusively by Ca^{2+} entering through L-type Ca^{2+} channels, and that Ca^{2+} released during a spark does not in turn trigger further activation of adjacent release sites. Although this is an appealing concept, recent studies provide evidence that it is not entirely correct. Spontaneous Ca^{2+} sparks could involve multiple sites of SR Ca^{2+} release[11,12]. This is only revealed using line-scans across the width of the cell (ie transversely, in the plane of the 'Z' discs); longitudinal scans showed no synchronization between the spontaneous sparks that arise at adjacent 'Z'-lines, 1.8 μm apart. Spontaneous Ca^{2+} sparks in cat atrial cells also can involve multiple sites[13].

As explained above, the fact that sparks have multiples sites of origin is potentially important for the theory of 'local control', since triggering of adjacent release sites during normal E-C coupling could lead to the loss of control of SR Ca^{2+} release by the Ca^{2+} current. The aim of the present study, therefore, was to determine if Ca^{2+} sparks evoked during E-C coupling can also involve multiple sites of origin, as do spontaneous sparks. We again used transverse scanning, but we evoked sparks by small depolarizing voltage-clamp pulses, so as to achieve a low probability of L-type Ca^{2+} channel opening and thus a correspondingly low probability of spark occurrence[3].

MATERIALS AND METHODS

Cell preparation and recording conditions Two-month-old Sprague-Dawley rats (200-300 g, from Medical Center of University of Maryland at Baltimore) were anesthetized with sodium pentobarbitone (17 mg/kg, ip). The hearts were removed from the animals via mid-line thoracotomy, and single ventricular cells were obtained by an enzymatic technique described in detail previously[4]. The cells were loaded with the Ca^{2+} indicator Fluo-3 by incubation for 30 min or longer in Tyrode's solution to which 5 μm Fluo-3 AM was added (Molecular Probes Inc, Eugene, OR, USA). Recordings of Ca^{2+} sparks were made in normal Tyrode's solution (composition in mmol/L: NaCl 140, dextrose 10, Hepes 10, KCl 4.0, $MgCl_2$ 1, $CaCl_2$ 1, pH adjusted to 7.3-7.4 with NaOH) at room temperature. Whole cell currents were recorded by standard methods. The electrode-filling solution was Na^+ free and was composed of (mmol/L): cesium glutamate 130, Hepes 10, TEA-Cl 20, $MgCl_2$ 0.33, Mg_2ATP 4, Fluo-3 (pentapotassium salt) 0.1; pH adjusted to 7.2-7.3 with CsOH. Electrodes had resistances of 1-4 MΩ. The holding potential was -45 mV. Current was digitized at 2 kHz with 12-bit resolution.

Fluorescence imaging Cells were imaged through a cover glass in the base of the recording chamber using a Nikon Diaphot inverted microscope (Nikon Inc, Melville, NY, USA) equipped with a 60x plan-apo oil-immersion objective (numerical aperture 1.4). All data were obtained by use of a 'homemade' line-scan confocal imaging system[14] attached to the camera port of the microscope. Briefly, excitation of Fluo-3 was achieved using the 488 nm line from a 100 mW argon-ion laser (Omnichrome, Chino, CA, USA), attenuated to 1 %-10 %, beam-expanded to overfill the back aperture of the objective, and deflected by a galvanometer-driven scan mirror (Cambridge Technology, Watertown, MA) positioned at a conjugate telecentric plane formed by an eyepiece lens placed in the camera port. Fluorescence emission was descanned by the same mirror and wavelengths >510 nm were directed onto a confocal aperture just large enough to encompass the central peak of the Airy disc diffraction pattern. Light passing the aperture was then focused onto the active area of an avalanche photodiode photon counting module (EG & G Canada Inc, Vaudreuil, Quebec, Canada), which produced TTL output pulses corresponding to each detected photon. Pulses from the detector were low-pass filtered at 30 kHz, to produce an analogue representation of fluorescence intensity along the scan line, and this was sampled at 10-ms intervals using the pClamp software package (Axon Instruments, Foster City, CA, USA) for continuous gap-free storage on disc. The fluorescence signal was also displayed on an oscilloscope as a function of distance along the scan line, providing immediate visual feedback about the spark activity. Computations and image analysis were performed on an IBM Risc System/6000 workstation (IBM, Armonk, New York) with the software IDL (Research Systems, Boulder, Colorado)[15]. Fluorescence images and measurements are presented as a ratio (F/F_o) of the fluorescence at any given time relative to the resting fluorescence at that position on the line-scan, derived from an average over several lines during a quiescent period prior to stimulation.

Statistics Data were expressed as mean±SD.

RESULTS

Sparks evoked during small depolarizations

Fig 1A illustrates our basic experimental protocol. Myocytes were depolarized from -45 to -35 mV (upper traces) while simultaneously recording whole-cell current and line-scan images representing fluorescence ratio (F/F_o; proportional to $[Ca^{2+}]_i$) along the scan line as a function of time. The line-scan image was formed by repeatedly scanning the laser spot along a 20 mm line oriented transversely across the width of the cell. Lines were scanned every 3 ms, and successive lines were stacked left to right, so that position along the line was depicted vertically and time ran horizontally. Each pixel displayed the ratio of the fluorescence relative to that at the same position during a quiescent period before the flash (F/F_o), and increasing fluorescence ratio (increasing free $[Ca^{2+}]$) was denoted by increasingly 'warm' colors as indicated by the bar. Whole-cell currents were typically small, on the order of 50 pA, and displayed little inactivation. Consistent with this, sparks occurred at a roughly constant rate during the pulse, with a probability about 10-fold greater than at the holding potential (Fig 1B)[4]. Thus, only a small proportion (ca 10 %) of sparks during depolarization would represent 'spontaneous' events. On the other hand, this depolarization gave a sufficiently low probability of spark occurrence to allow clear resolution of individual events, and to minimize the chance of near-simultaneous open-

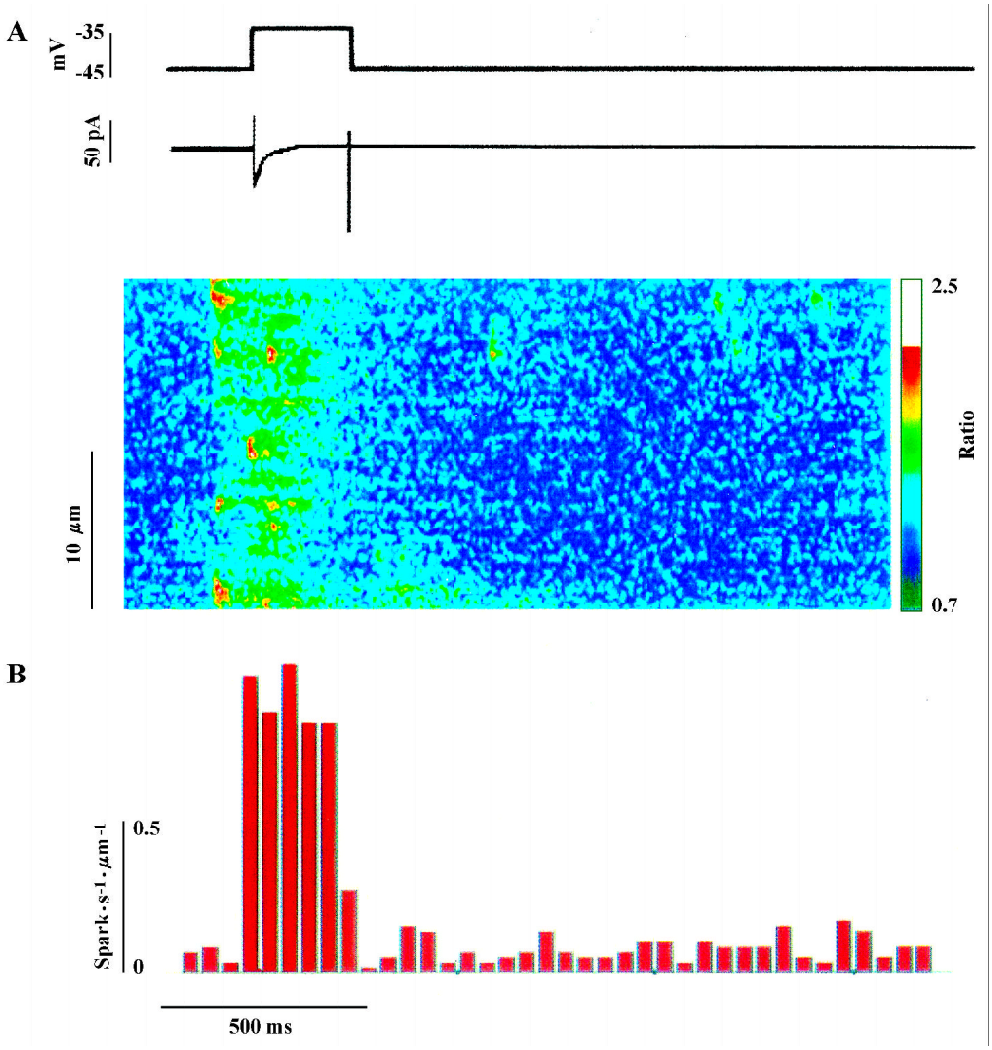

Fig 1. Linescan imaging of sparks evoked with low probability during small voltage-clamp depolarizations. (A) Records of clamp potential (upper) and whole-cell Ca^{2+} current (lower) obtained simultaneously with a transverse line-scan image of spark activity (color image). (B) Mean numbers of sparks during successive 50-ms intervals throughout the voltage-clamp pulse protocol. Data show total numbers of sparks per 50 ms observed during 6 repeated trials in each of 11 cells. The mean spark probability during the pulse was about 10-fold greater than that at the holding potential.

ings of L-type channels at neighboring release sites. For the data shown, the rate of spark occurrence increased during the depolarizing pulse to about 1 spark· s^{-1}· μm^{-1} (transverse scan). Some of the sparks evoked during depolarization appeared, in transverse line-scan images (Fig 1A) to involve multiple sites.

Representative examples of individual evoked sparks recorded by transverse line scanning were shown in Fig 2A, B, where the fluorescence ratio is represented both by color and by height of the surface. Similar to our previous findings with spontaneous sparks[11], some evoked sparks (Fig 2B) appeared to arise at two discrete sites. This was evident both by separate peaks of fluorescence along the scan line and by a greater overall spatial spread of the Ca^{2+} signal as compared to events that appeared to involve only a single site (Fig 2A). In some other cases, however, it was not possible to clearly resolve separate peaks in the signal. As a less subjective method by which to estimate the proportions of sparks involving single or multiple sites, we measured the full-widths (in μm) of sparks at their half-maximal amplitudes (FWHM) at the time of peak fluorescence (Fig 2C, 2D). This parameter would include both the diffusive spread of Ca^{2+} and, for multiple-site events, the separation between release sites along the scan line.

Spontaneous and evoked sparks involving multiple sites The distributions of FWHM measurements for both spontaneous (upper) and evoked sparks (lower) in cells examined by either longitudinal scanning (left; 6 cells) or transverse scanning (right; 11 cells). Histograms were fit by single or multiple Gaussian distributions (Origin 4.1; Microcal Software). In longitudinal

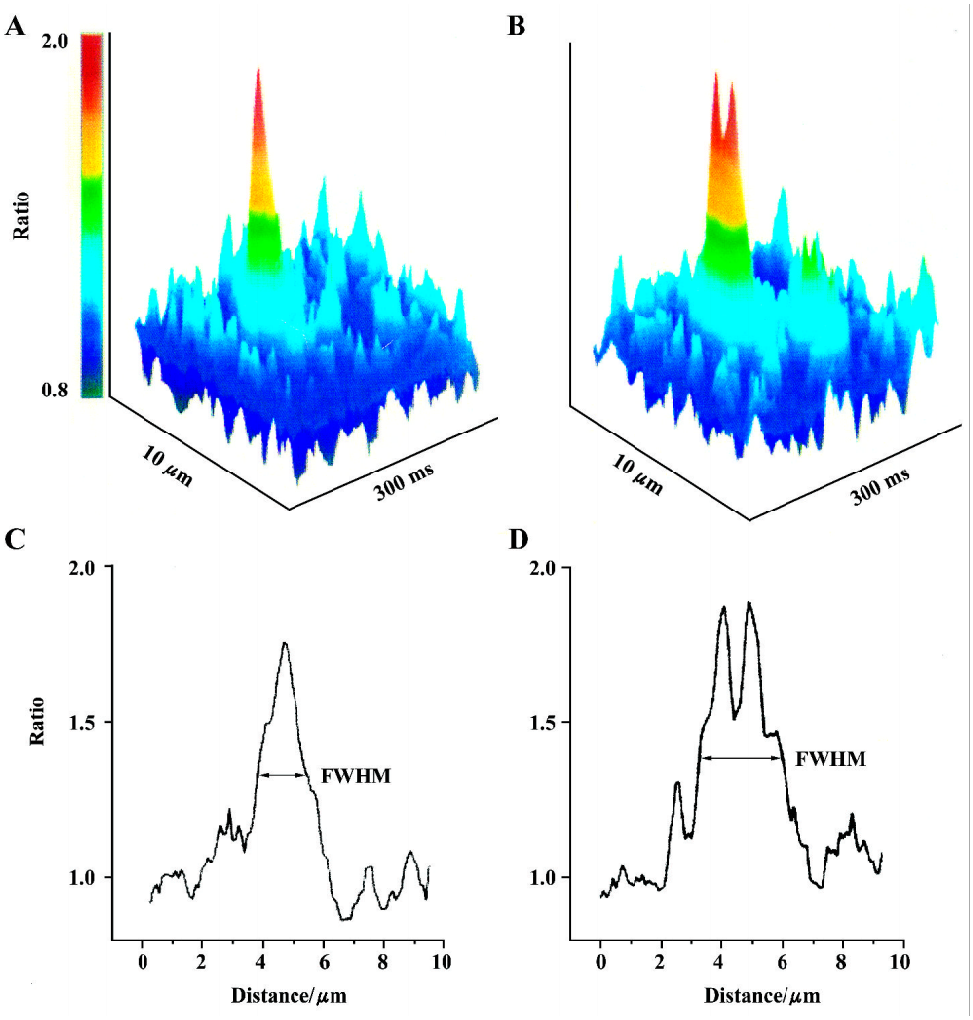

Fig 2. Illustrations of sparks recorded by transverse line scanning which showed single and double sites of origin, and method of measuring spark width. The spark in (A) appeared to arise at a single site, whereas that in (B) showed two distinct peaks. C, D, profiles showing fluorescence ratio along the scan line of these sparks at the times of peak fluorescence. Arrowed lines illustrate measurement of the full width at half maximal amplitude (FWHM).

scans, the distributions of spark widths for both spontaneous (A) and evoked sparks (C) were described well by single Gaussian functions with virtually identical means and standard deviations respectively (2.6 μm±0.4 μm and 2.6 μm±0.5 μm). Different to this, transverse scans showed bimodal distributions of spark widths for both spontaneous (B) and evoked events (D). These distributions were each fit by the sum of two Gaussian functions with the following means and standard deviations: spontaneous sparks, 1.8 μm±0.8 μm and 2.9 μm±0.5 μm; evoked sparks, 1.9 μm±1.0 μm and 3.1 μm±0.8 μm. Relative areas under the two Gaussians were 1.73:1 and 1.85:1, respectively, for spontaneous and evoked sparks (Fig 3).

The Gaussian distributions of FWHM measurements in longitudinal line scan were thus consistent with sparks arising independently from single release sites along the long axis of the cell. In the transverse direction, however, the bimodal distributions suggested two populations of sparks, arising at either single sites or at paired sites separated by a mean distance of roughly 1.2 μm. For both spontaneous and evoked sparks the double-site events represent about one-third of the total population, and there was also evidence of a few sparks (3 % of total) possibly involving three sites (Fig 3B,D). The mean width of presumed single-site sparks measured in transverse scans (1.8-1.9 μm) was less than that of sparks in longitudinal scans (2.6 μm), but this is in agreement with the previously reported anisotropy of Ca^{2+} diffusion in the myocyte[1,11].

DISCUSSION

Our data indicated that Ca^{2+} sparks evoked by depolarization (presumably by voltage-activated L-type Ca^{2+} currents) could have multiple sites of origin along the Z-lines. Under our conditions, a little over one-third of sparks appeared to arise at two or more sites; and this figure is probably an underestimate, since the line scan images would not have resolved axially displaced sites (ie, above or below one another). One possibility is that ‘ double’ sparks arose through independent trig-

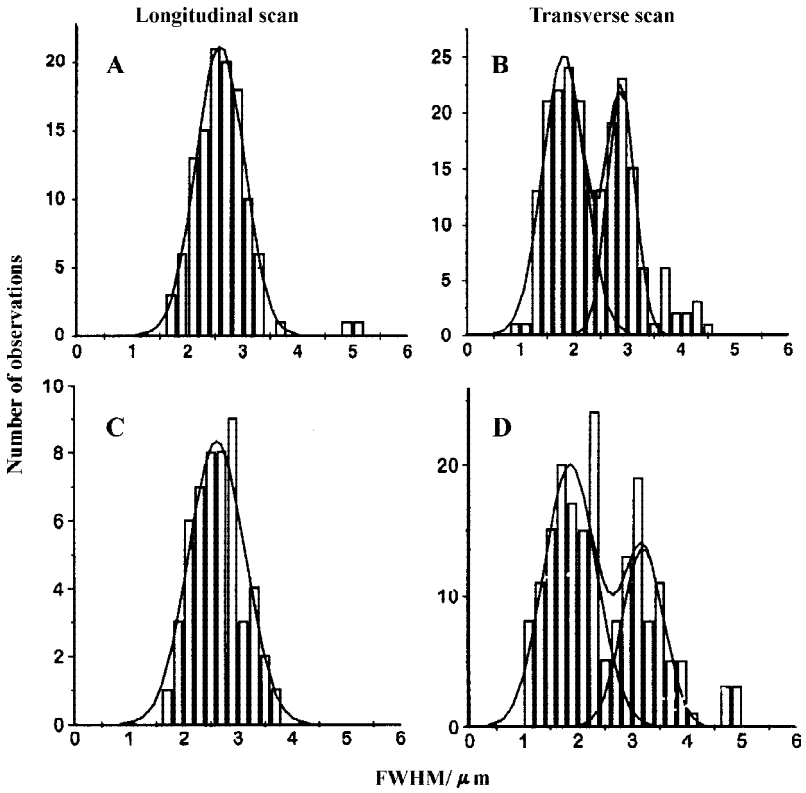

Fig 3. Measurements of spark widths for both spontaneous and evoked responses show normal distributions in longitudinal line-scans, but bimodal distributions in transverse scans. Histograms show distributions of spark widths (FWHM) for (A) Longitudinal scans of spontaneous sparks (6 cells, 166 sparks); (B) Transverse scans of spontaneous sparks (11 cells, 204 sparks); (C) Longitudinal scans of evoked sparks (6 cells, 61 sparks); (D) Transverse scans of evoked sparks (11 cells, 191 sparks).

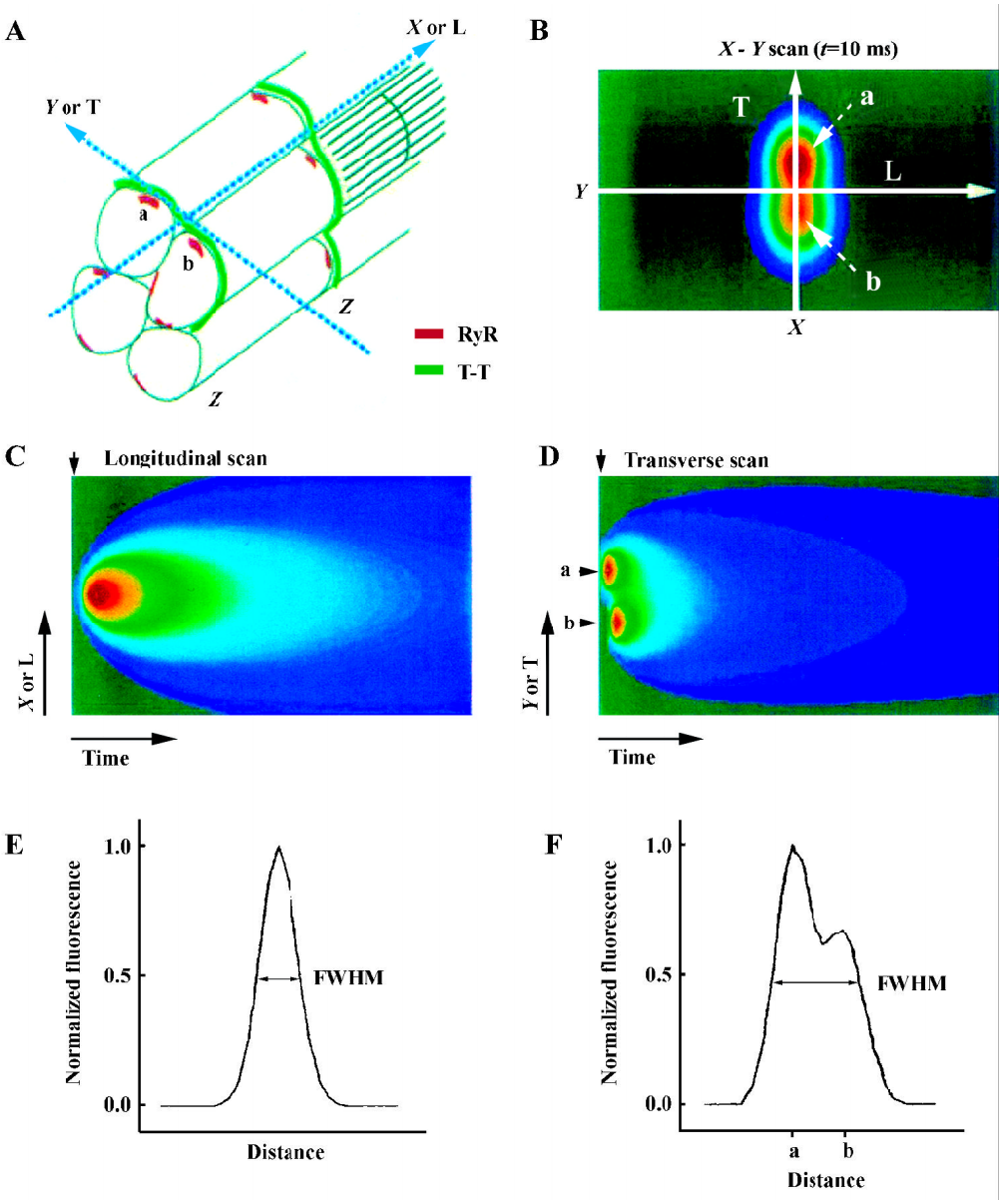

Fig 4. Scheme illustrating the proposed arrangement of Ca^{2+} release sites in the myocyte, and the resulting patterns of Ca^{2+} distribution that would be observed by different imaging methods. (A) Schematic diagram showing locations of Ca^{2+} release sites (red) and T-tubules (green) in relation to myofibrillar bundles and Z-discs. Dashed lines (blue) indicate orientations of longitudinal (*X* or L) and transverse (*Y* or T) scan lines used to derive the representative images shown in (B-D). (B) Two-dimensional scan (*X*-*Y*) showing two peaks of fluorescence arising from Ca^{2+} release at the sites denoted ‘ a’ and ‘ b’ in A. The image is a ‘ snapshot’ captured at the peak of the signal at ‘ a’. (C) A longitudinal (*X* or L) image along a line midway between the two sites fails to resolve the multiple sites of origin. (D) Transverse scanning along a line intersecting ‘ a’ and ‘ b’ clearly reveals Ca^{2+} release from two sites following different latencies. (E and F) Respective fluorescence profiles through the line-scan images in C and D, obtained the time of maximum fluorescence at site ‘ a’, and illustrating the increased width of the multiple-site spark in a transverse scan.

gering by the chance, near-simultaneous opening of L-type Ca^{2+} channels at neighboring sites. For several reasons, however, this is unlikely to be the case. Firstly, the probability of spark occurrence was low during the depolarizations to -35 mV (ca 1 spark· s^{-1}· μm^{-1}) and the resolution of our recordings was such that only independent sparks occurring within less than about 10 ms of one another would have been counted as single events. The proportion of events where a spark at one site was accompanied within 10 ms by an independent spark at a neighboring site on either side would, therefore, be <1 % much smaller than the observed occurrence of

>33 % ‘ double’ sparks. Secondly, longitudinal line scan images did not show any instances of evoked sparks involving synchronous release at adjacent Z-lines although the probability of near-simultaneous openings of L-type channels would presumably be the same for sites displaced longitudinally as well as laterally. Finally, the relative proportions of sparks showing multiple sites of origin were closely similar for both evoked and spontaneous events. This indicates strongly that the same phenomena that allow multiple sites of origin in spontaneous sparks allow multiple sites for evoked sparks.

Our hypothesis to explain these results is illustrated in Fig 4. A schematic showing several bundles of myofibrils and associated Ca^{2+} release sites (clusters of RyR) are shown in (A), together with the longitudinal (L or ‘*X*’ axis) and transverse (T or ‘*Y*’ axis) orientations of our line scans. We assume that release of Ca^{2+} from RyR at a T-tubule-SR junction (eg, site ‘ a’) is evoked first by Ca^{2+} entry through a tightly apposed L-type Ca^{2+} channel. The Ca^{2+} released from the SR spreads by diffusion, and may trigger release of Ca^{2+} from a closely neighboring site (‘ b’) within the plane of the same Z-disc, but not from more distant sites 1.8 μm away in the adjacent Z-discs[11]. Two-dimensional (*X*-*Y*) imaging would thus clearly reveal the two sites of origin (Fig 4B); but this is not technically feasible with our present instrumentation. Linescan imaging provides only more limited information, and in particular, a longitudinal scan (eg, along the lines indicated in A and B) would give no clue of the two sites of origin (Fig 4C). On the other hand, a transverse scan through the sites ‘ a’ and ‘ b’ directly reveals the two sites of origin (Fig 4D), and a fluorescence profile taken at the peak of the spark at site ‘ a’ (Fig 4F) shows two unequally sized peaks (due to a lag in activation at site ‘ b’) with an overall FWHM greater than that observed during longitudinal scanning (Fig 4E).

On the basis of these results we propose the following modifications to the ‘ local control’ theory of E-C coupling-though its central tenet of independent activation of domains of Ca^{2+} liberation by single L-type channels remains unchanged. Firstly, the data further strengthen the view that evoked sparks arise through release of Ca^{2+} from a cluster of RyR, rather than an individual RyR, even when the spark appears to originate at a single site. Electron microscopic studies indicate that RyR are organized into clusters some tens of nanometers in size[16], and it seems improbable that Ca^{2+} released through one channel would fail to activate other channels within the same cluster while still triggering release at much more distant sites as much as 1 μm away. Secondly, the small and brief Ca^{2+} flux through an open L-type channel appears able to directly trigger only its immediately apposite release site, whereas the greater amount of liberated Ca^{2+} may subsequently trigger release at neighboring sites. Finally, this functional coupling between adjacent release sites increases the overall gain of E-C coupling, and indicates that the ‘ independent Ca^{2+} release unit’ [17] is not a single RyR or tight cluster of RyR, but may involve several discrete sites at m spacing within Z-discs. The factors that limit the spread of activation of release in the transverse direction are not known, but presumably include the geometrical disposition of T-tubule/SR junctions, the apparent diffusion coefficient for Ca^{2+} and the relative excitability of release sites. Longitudinally, release sites are too far apart to allow coupling of activity, except under conditions of Ca^{2+} overload. However, it remains to be determined whether the probability of ‘ transverse coupling’ changes with experimental conditions.

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