Variability in frequency and characteristics of Ca^{2+} sparks at different release sites in rat ventricular myocytes

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1. High spatial resolution confocal imaging was used to investigate spontaneous calcium release events (Ca^{2+} sparks) in isolated rat cardiac myocytes loaded with the fluorescent calcium indicator fluo-3.

2. Frequencies of sparks at different release sites varied widely, with a few sites showing sustained activities as great as 50 times the average. Sites with frequent sparks showed more rapid recovery of activity following Ca^{2+} waves and locally elevated basal [Ca^{2+}].

3. In addition to transient sparks, some sites showed bursts of rapid flurries of spark-like events, or apparently sustained Ca^{2+} liberation. Bursts remained localized to individual z-lines, but adjacent sites on the same z-line could be 'driven' by a bursting site to generate similar activity.

4. Individual sites showed long-term (tens of seconds) changes in 'modes' of activity, with abrupt transitions in frequencies of sparking, and between transient sparks and sustained bursts. These transitions were not associated with changes in the amplitude of the sparks.

5. We conclude that spontaneous sparks are not stereotyped events generated with uniform probability at all sites. Instead, the Ca^{2+} release event is variable, and some sites have a high probability of spark generation. Both factors show long-term changes at individual sites, raising the possibility that properties of fundamental Ca^{2+} release units may be subject to modulation.

Calcium sparks are localized subcellular changes in intracellular free calcium ion concentration ([Ca^{2+}]) that occur spontaneously in mammalian heart cells (Cheng, Lederer & Cannell, 1993) and are evoked also by Ca^{2+} influx through L-type Ca^{2+} channels (López-López, Shacklock, Balke & Wier, 1994, 1995; Cannell, Cheng & Lederer, 1994, 1995) during action potentials or voltage-clamp depolarization. Ca^{2+} sparks are thought to represent elementary units of Ca^{2+} release (Bootman & Berridge, 1995) and arise through Ca^{2+} liberation from the sarcoplasmic reticulum (SR) at sites associated with T-tubules, spaced at regular intervals about 1·8 μm along the length of the cell (Shacklock, Wier & Balke, 1995). The prevailing local control theory of excitation–contraction coupling (Stern, 1992) holds that sparks are 'recruited' independently of each other, during large L-type Ca^{2+} currents, to produce the whole-cell Ca^{2+} transient. This concept helps account for the graded control of Ca^{2+} release with depolarization, despite the inherently regenerative nature of Ca^{2+}-induced Ca^{2+} release (CICR) from the SR.

The fundamental nature of the calcium spark remains unknown. Cheng and his colleagues (Cheng *et al.* 1993) suggested that calcium sparks arise from the release of Ca^{2+} through individual channels (ryanodine receptor, RyR), but the alternative possibility – that calcium sparks involve more than one channel 'acting in concert' (Cheng *et al.* 1993) – has never been eliminated. In that regard, we (Parker, Zang & Wier, 1996) showed recently that Ca^{2+} sparks can involve Ca^{2+} release at multiple closely packed sites along z-lines in rat heart cells, and Lipp & Niggli (1996) proposed that Ca^{2+} flux through individual channels gives rise only to Ca^{2+} signals (quarks) much smaller than the sparks. With respect to the properties of Ca^{2+} sparks, it has been shown that the amplitudes, time courses, and overall frequency of occurrence of Ca^{2+} sparks in rat ventricular myocytes changes with the concentration of Ca^{2+} (1 mM and 10 mM) in the external solution (Cheng, Lederer, Lederer & Cannell, 1996). It has also been shown that the probability of evoked sparks is the same in successive segments of cell length of 1·8 μm during voltage-clamp ramps (Cannell *et al.* 1995). A

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different study (Shacklock *et al.* 1995) reported that a few *z*-lines appeared to have a somewhat higher than average probability of generating sparks during depolarization. Nevertheless, there has been no information on how the frequency, amplitude, and time course of Ca^{2+} sparks might vary (with or without elevated external $[Ca^{2+}]$) at a single sparking site or on how activity might vary from site to site, or on how activity might vary over time, at a single, identified site. By using a homemade line-scan confocal microscope (Parker *et al.* 1996; Parker, Callamaras & Wier, 1997) which is able to record continuously for long periods, we now show that spontaneous activity at different release sites varies widely. Some sites generate sparks at frequencies as great as 50 times the average, and others show prolonged steps of apparently continuous Ca^{2+} liberation, rather than transient sparks. Furthermore, individual sites show long-term (tens of seconds) changes in modes of activity, raising the possibility that properties of functional release units may be subject to modulation rather than invariably generating the same all-or-none signal.

METHODS

Cell preparation and recording conditions

Two-month-old Sprague–Dawley rats (200–300 g) were anaesthetized with sodium pentobarbitone (17 mg kg^{-1} I.P.). 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 (López-López *et al.* 1995). The cells were loaded with the Ca^{2+} indicator fluo-3 (Minta, Kao & Tsien, 1989) by incubation for 30 min or longer in Tyrode solution (composition (mM): NaCl, 140; dextrose, 10; Hepes, 10; KCl, 4·0; $MgCl_2$, 1; $CaCl_2$, 1; pH adjusted to 7·3–7·4 with NaOH) to which 5 mM fluo-3 AM was added (Molecular Probes Inc., Eugene, OR, USA). Recordings were made at room temperature (23 °C), with cells placed in a recording chamber constructed using a coverslip as its base, and bathed in Tyrode solution with $[Ca^{2+}]_o$ between 0·5 and 2·0 mM so as to achieve a convenient frequency of sparks while minimizing the occurrence of Ca^{2+} waves.

Fluorescence imaging

Cells were imaged using a Nikon Diaphot inverted microscope (Nikon Inc., Melville, NY, USA) equipped with a ×60 plan-apo oil-immersion objective lens (numerical aperture, 1·4). With the exception of Fig. 2*D*, all data were obtained by use of a homemade line-scan confocal imaging system (Parker *et al.* 1996; Parker, Callamaras & Wier, 1997) attached to the camera port of the microscope. 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 lens, and deflected by a galvanometer-driven scan mirror (Cambridge Technology, Watertown, MA, USA) 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 of greater than 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 transistor–transistor logic output pulses corresponding to each detected photon. The scan mirror was driven by a saw-tooth waveform, adjusted so that the laser spot formed in the specimen repeatedly scanned a line 20 μm long every 3 ms. Fluorescence excited by this line in the specimen could be directly viewed together with a brightfield image of the cell through a laser-line blocking filter in the microscope eyepiece, thus facilitating alignment of the scan either along the length of the cell or transversely. 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 μs intervals using the pCLAMP software package (Axon Instruments, Foster City, CA, USA) for continuous gap-free storage on disk. 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, and thus facilitating location of highly active sites.

RESULTS

Certain sites generate sparks at high frequency

Images produced by scanning myocytes longitudinally showed patterns of elevated background fluorescence at regular spacings of about 1·8 μm, which correspond to the positions of T-tubule–SR junctions (*z*-lines) and are sites of spark generation (Shacklock, *et al.* 1994; Parker *et al.* 1996). Most such sites showed infrequent sporadic sparks but a small proportion of sites (e.g. sites marked by arrows in Fig. 1*A*) in some line-scan images produced a much higher frequency of spark occurrence. Study of these 'frequent' sites was facilitated in the present experiments by the ability to view fluorescence activity in real time on the oscilloscope screen and thus more easily locate frequent sites. Once located, the position of the scan line was adjusted (in the *y*- and *z*-axes) to record as close to the site of Ca^{2+} release as possible (i.e. to maximize spark amplitudes). Figure 1*A* and *B* illustrates the time course of fluorescence at such a site, together with infrequent sparks at other sites in the image and a spontaneous Ca^{2+} wave.

Figure 2*A* shows the observed distribution of numbers of sites generating a given number of sparks during 12 s recording periods. Data were compiled from several longitudinal line-scan images in different cells, comprising a total of 350 *z*-lines. Roughly one-half of all *z*-lines failed to give even a single spark during each image, although most, if not all, represent functional release sites, since the radial density of release sites is high and sparks are observed at virtually every *z*-line if observed for sufficiently long times (Parker *et al.* 1996). About one-fifth (67) of the sites showed one spark, and the proportions of sites showing increasing numbers of sparks decreased progressively. However, the distribution showed a 'tail', with a significant number of sites showing as many as 50 sparks $(12\ s)^{-1}$ (4 sparks s^{-1}). The question then arises as to whether particular sites have an inherently greater than average probability of generating sparks, or whether the apparent high frequency resulted from statistical variation among a population of sites all having an equal and low probability of spark generation. From Fig. 2*A*, the mean number of sparks per site per 12 s period (excluding the tail of sites showing > 10 sparks) was

1·12. If all sites generate sparks with equal probability, a Poisson distribution with this mean predicts a vanishingly small probability (0·0001) of observing even a single site showing 10 sparks or more among the 350 *z*-lines imaged. Very different to this, twenty-five such sites were actually observed. The occurrence of sparks at individual sites is, therefore, not adequately described by a completely stochastic (Poissonian) process (cf. Shacklock *et al.* 1995) and, in particular, it is highly improbable that the high frequencies of sparks at some sites arose simply from statistical variation. This conclusion is unaffected by the deliberate selection during experiments of sites showing high frequency sparks because, if spark generation were stochastic with a uniform mean occurrence rate, frequent activity during the selection period would have no bearing on subsequent activity while recording. Thus, a small proportion of release sites appear to show a much higher than average rate of spark generation ('frequent' sites). It should be noted, however, that the occurrence of such sites is certainly overestimated in Fig. 2 as a result of selection – probably by a factor of 10–100.

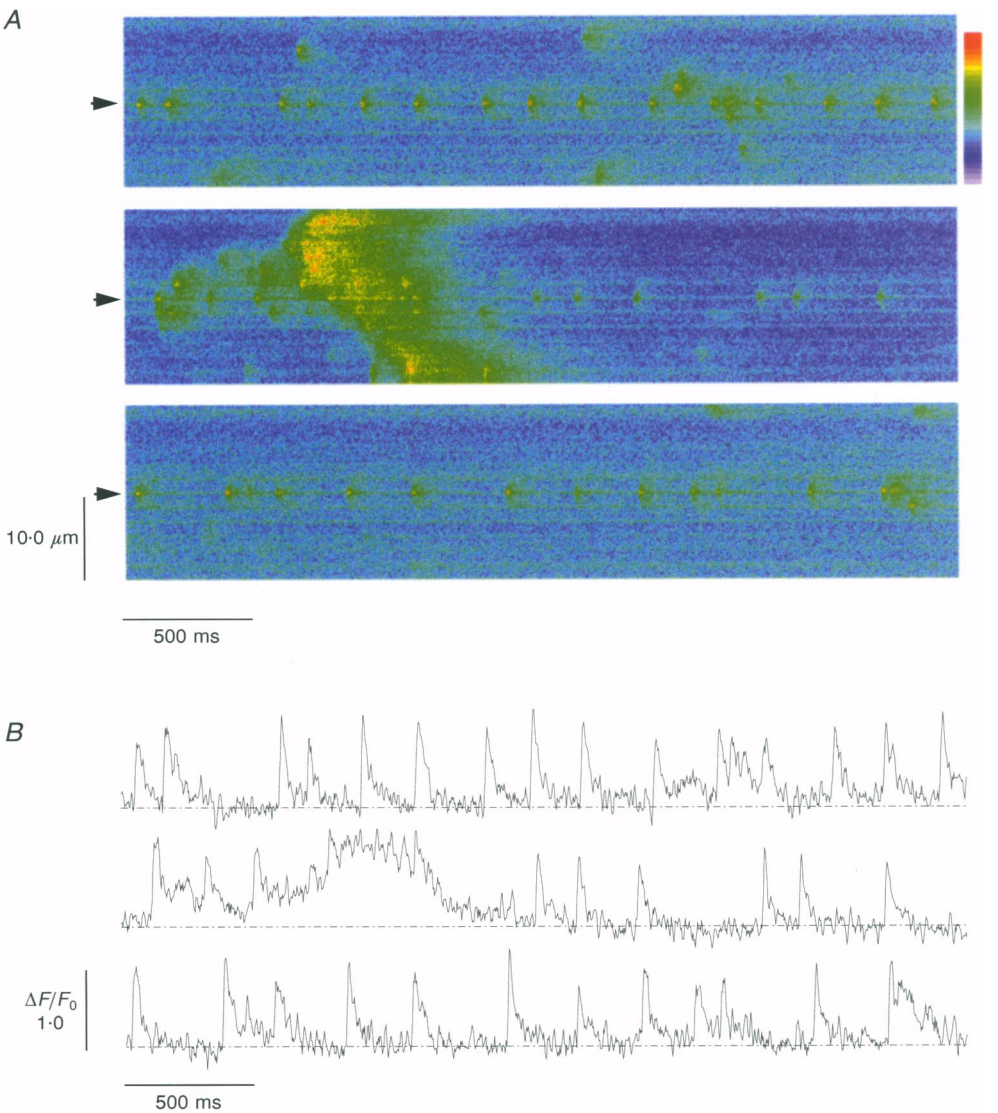

Figure 1. Frequent sparks originating at a single site

A, line-scan images show Ca^{2+}-dependent fluorescence recorded by repeatedly scanning along a 20 μm line oriented along the length of a ventricular myocyte at 3 ms intervals. Position along the line is depicted vertically, and successive lines are stacked left to right. Increasing fluorescence is depicted by increasingly warm colours, coded as indicated by the colour bar. The three frames form a contiguous record. Faint bright lines correspond to positions of T-tubule–SR junctions (*z*-lines). The site marked by the arrowheads showed frequent sparks, and a spontaneous Ca^{2+} wave crossed the cell during the second frame. *B*, fluorescence signals monitored from a single pixel at the corresponding positions (top to bottom) marked by the arrowheads in *A*. Calibration bar corresponds to a fractional increase in fluorescence above the resting baseline ($\Delta F/F_0$) of 1·0.

Intervals between sparks at frequent sites

Sparks at sites showing infrequent activity tended to occur at irregular intervals (Figs 1*A* and 3*A*; and see, for example, Shacklock *et al.* 1995; Parker *et al.* 1996). This would be consistent with a stochastic (random) triggering of Ca^{2+} release (Cannell *et al.* 1995), although the small numbers of events during even long records precluded quantitative analysis. Conversely, sparks at highly active sites occurred with a more regular periodicity (Fig. 1*A* and *B*), suggesting that the minimal interval between sparks may be further determined by a refractory period following each event. Measurements of intervals between successive sparks at a single site (Fig. 2*B*) showed that most sparks occurred after intervals of 150–200 ms, with an abrupt fall-off at shorter intervals, and a more gradual, roughly exponential decline at progressively longer intervals. Similar results were obtained from a further three frequent sites. Thus, similar to factors determining intervals between inositol 1,4,5-trisphosphate ($Ins P_3$)-mediated Ca^{2+} 'puffs' (Yao, Choi & Parker, 1995), inter-spark intervals appear to be determined both by recovery from a refractory state established by the preceding spark, and a stochastic triggering which leads to an exponential distribution at longer intervals.

The amplitudes of sparks occurring after short intervals might be expected to be reduced if there were insufficient time for complete refilling of the Ca^{2+} store, or if a proportion of the channels involved in the release remained refractory. To look for such effects, we plotted the peak amplitudes of sparks as a function of interval following each preceding spark. In records from four cells (total of 150 sparks) we found no significant correlation, indicating that any changes in spark amplitude resulting from closely preceding sparks were small (< 10%) and negligible in comparison with other sources of variability.

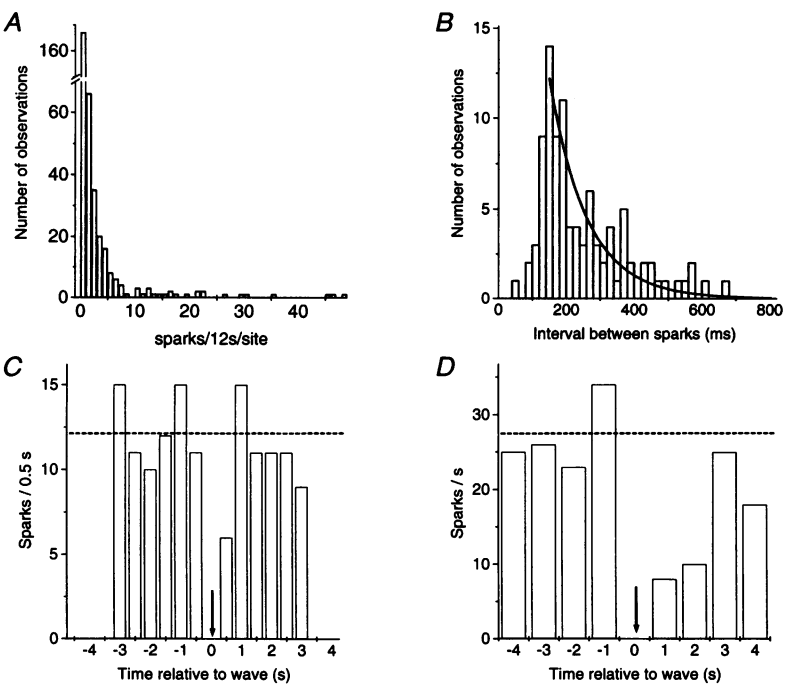

Figure 2. Occurrence of sites showing frequent sparks, and analysis of factors affecting intervals between successive sparks

A, distribution of numbers of spark sites observed to generate a given number of sparks during a 12 s recording period. Data were compiled from 32 longitudinal line-scan images encompassing a total of 350 spark sites. The number of failures (sites showing no sparks) was obtained by summing the numbers of *z*-lines which failed to show sparks during the recordings. *B*, distribution of intervals between successive sparks at a single high frequency site. Continuous curve shows an exponential of time constant 120 ms fitted to the data at intervals > 140 ms. *C*, changes in spark frequency at frequent sites following Ca^{2+} waves. Histogram shows total numbers of sparks observed during 0·5 s intervals preceding and following spontaneous Ca^{2+} waves, pooled from observations of 6 waves. Arrow marks time of the waves, and no measurements of spark frequency were made during this interval due to the difficulty in discerning sparks during the wave. Dashed line indicates mean number of sparks per interval preceding waves. *D*, similar analysis of effect of waves on frequencies of sparks at 'normal' sites. Data were derived from video fluorescence records of single myocytes, and histogram shows total number of sparks during 1 s intervals among 5 cells.

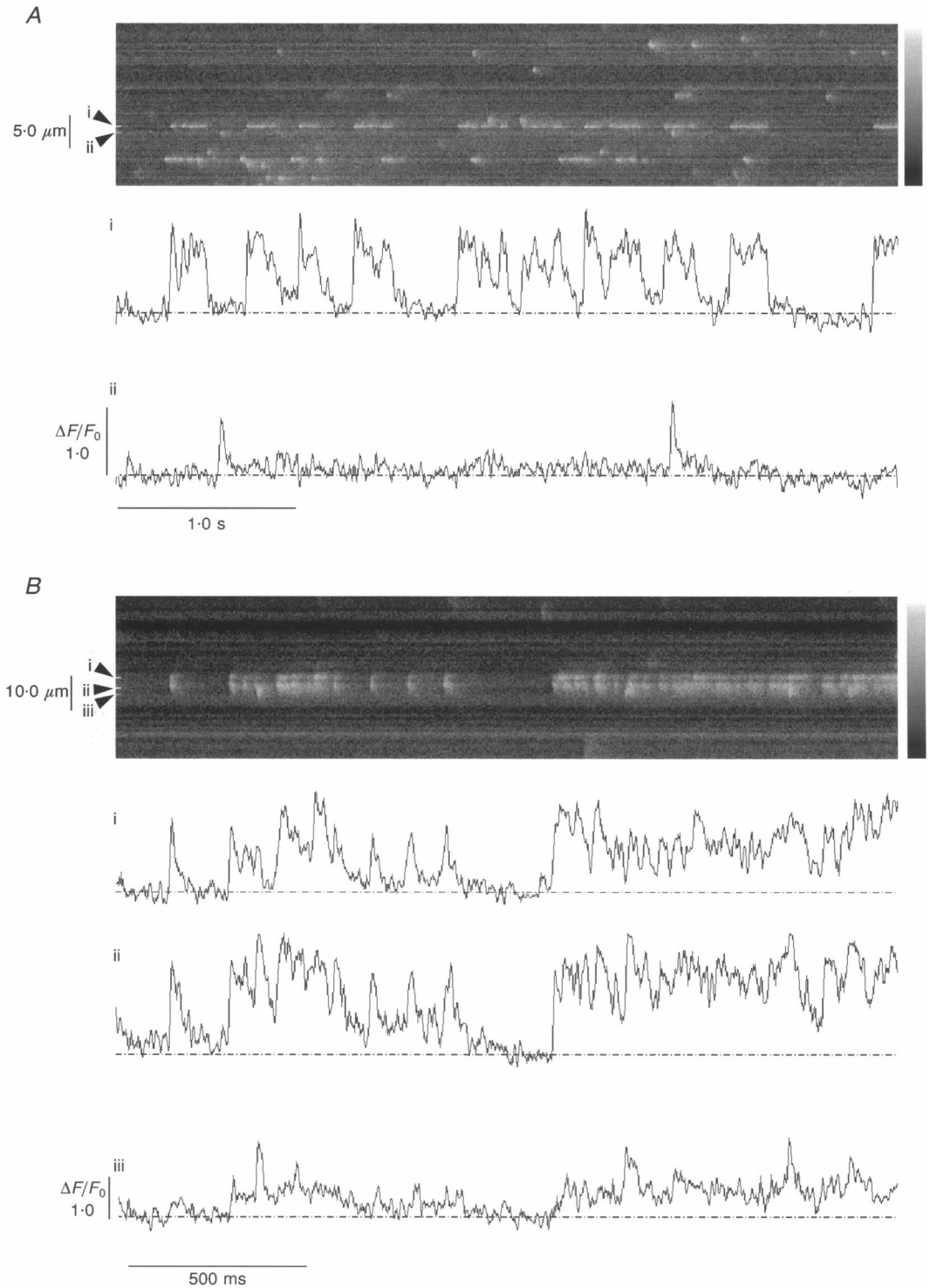

Figure 3. Bursting activity is localized to single sites along the length of the cell, but may involve synchronous activation of adjacent transverse sites

A, longitudinal line-scan image shows independent bursting activity at 2 sites and transient sparks at other sites. Traces show fluorescence monitored from a single pixel at the bursting site (i) marked by the arrowhead in the image and independent sparks monitored at an adjacent site (ii). *B*, line-scan image was obtained scanning transversely across a cell and shows coupled bursting activity at 2 adjacent sites (i and ii), together with independent sparks at a third, closely neighbouring site (iii). Traces below show fluorescence monitored at single pixels corresponding to the sites (i–iii) in the line-scan image.

Inhibition of sparks by waves

The higher activity at frequent spark sites might arise because of more rapid recovery from a refractory state (Gyorke & Fill, 1993; Cheng *et al.* 1996), or because the probability of triggering of sparks is higher than at infrequent sites. It has been shown that the ability to evoke release is depressed after a wave (Cheng *et al.* 1996). To look for differences in recovery from inhibition, we measured changes in spark frequency following spontaneous Ca^{2+} waves. Figure 2*C* shows pooled data of the numbers of sparks at frequent sites during 0·5 s time bins before and after waves, derived from line-scan images like Fig. 1*A*. Because of the elevated fluorescence during the wave, spark frequencies could not be estimated during that time bin, but during the following 0·5 s interval the spark frequency was depressed to about one-half the initial level before the wave. However, the frequency then recovered rapidly, and returned to control levels within 1 s following the wave. Similar data from infrequent sites were difficult to obtain from line-scan images, since relatively few sites are encompassed by the line-scan. We therefore made analogous measurements from widefield video-fluorescence images (Fig. 2*D*), which sample from a much greater volume of the cell (Parker & Wier, 1996*a*). The occurrence of sparks at these sites remained depressed for longer times following waves, recovering by only about 40% of the control level after 2 s. Thus, a faster recovery from inhibition may account, at least partly, for the ability of certain sites to generate sparks at a relatively high frequency.

Bursting modes of spontaneous Ca^{2+} release

In addition to the generation of sparks at widely differing frequencies, a further pattern of activity seen at some sites was the generation of prolonged (hundreds of milliseconds or several seconds) bursts of Ca^{2+} release (Figs 3 and 4*A*). In some cases these appeared to be composed of rapid flurries of spark-like events (Fig. 3*A*), whereas others showed apparently sustained, continuous Ca^{2+} elevations (Fig. 4). Cheng *et al.* (1993) described the appearance of similar events in the presence of low concentrations of ryanodine, and attributed them to ryanodine causing release channels to enter a low-conductance, long open lifetime state; but that was not the case in the present recordings obtained in the absence of pharmacological agents.

As noted above, Ca^{2+} release sites are spaced at regular intervals of about 1·8 μm along the length of myocytes, and

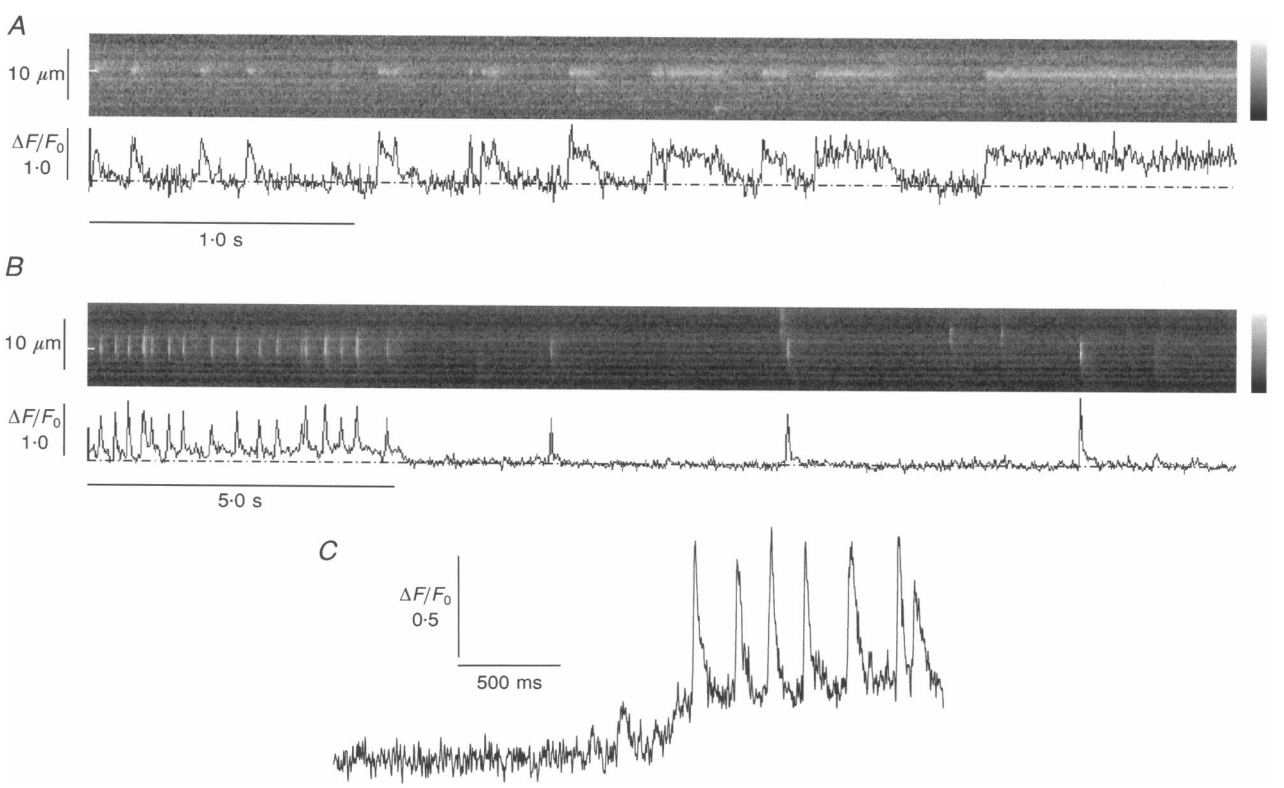

Figure 4. Patterns of spark activity at given sites show long-term changes in mode

A, longitudinal line-scan image illustrating a site showing a transition from transient sparks to prolonged Ca^{2+} release. Trace shows fluorescence monitored from a single pixel centered on the spark site (position marked by white bar in image). *B*, similar records in a different cell, showing an abrupt decrease in spark frequency. *C*, fluorescence monitored by a stationary laser spot, illustrating an abrupt increase in spark frequency following a rise in basal Ca^{2+} fluorescence.

sparks occur autonomously at different sites. This functional independence of longitudinally adjacent sites also held during bursts, despite the much longer durations of Ca^{2+} release, which might have been expected to increase the probability that Ca^{2+} diffusing from a release site would trigger CICR at an adjacent site. For example, a site immediately adjacent to the bursting site in Fig. 3*A* generated two transient sparks showing no obvious correlation with the bursting activity (lower trace, Fig. 3*A*). On the other hand, release sites are packed at higher density radially across the cell, and transverse line-scan images (parallel with the striations) reveal that adjacent pairs and triplets of sites often show near-synchronous sparks, with release at one site triggering others (Parker *et al.* 1996). Such behaviour was apparent also during bursts, as illustrated in Fig. 3*B*, which shows two transverse sites (i and ii) displaying highly correlated bursting activity. Because the activity at each site did not always exactly mirror the other (Fig. 3*B*, traces i and ii), and because a third site at a yet closer spacing showed quite independent sparks (Fig. 3*B*, trace iii), it is unlikely that this resulted artifactually because Ca^{2+} released from a single site gave rise to apparently discrete signals at two regions of high dye concentration. Instead, it appears that sustained release of Ca^{2+} at one site is sometimes able to evoke sustained Ca^{2+} liberation at a transversely adjacent site 1 μm or more away, rather than merely triggering more frequent transient sparks.

Modal changes in spark activity

Most recordings from high frequency sites showed sparks occurring at a rapid and roughly constant rate for tens of seconds. However, we observed a few instances when sites showed abrupt transitions from a low frequency of sparks to a sustained higher rate, or vice versa (Fig. 4*B* and *C*). In all cases ($n = 3$), periods of frequent sparking were accompanied by small elevations of basal Ca^{2+} level. This is shown most clearly in Fig. 4*C*, obtained using stationary point confocal microfluorimetry (Parker & Wier, 1996*b*) to provide an improved resolution of small Ca^{2+} changes. Because the changes in basal Ca^{2+} preceded and lagged the transitions in spark frequency by a few hundred milliseconds, it seems they did not arise simply as a result of greater net Ca^{2+} efflux during the sparks. Instead, local elevations of basal Ca^{2+} level may have driven the increases in spark frequency.

Transitions were also observed at individual sites between generation of transient sparks and bursts or periods of sustained Ca^{2+} liberation (Fig. 4*A*). Because these most often involved a progressive transition from transient sparks to bursts of increasingly long duration (e.g. Figure 4*A*), we were concerned whether this might arise through photodamage caused by the laser scan. However, two observations suggest this was not the case: (i) bursts usually involved only a single site, while other sites imaged by the laser scan continued to show transient sparks, and (ii) sites showing bursts sometimes reverted to generating transient sparks.

DISCUSSION

The great majority of release sites generate sparks at irregular intervals at a mean rate of 0·1 s^{-1} or less, and the numbers of sparks observed at different sites in a given time approximate a Poisson distribution (Shacklock *et al.* 1995), suggesting that sparks arise through a stochastic process at sites having similar probabilities of release. However, a very small number of sites produce, at least for brief periods, sparks at frequencies as high as 50 times that mean rate. Generation of high frequency sparks might arise from two factors: a faster recovery from a refractory state resulting from Ca^{2+} release during a preceding spark (Fabiato, 1985; Gyorke & Fill, 1993), or an increased probability of stochastic triggering. Evidence for a faster recovery from inhibition induced by Ca^{2+} waves was seen in the faster recovery of spark rate at frequent compared with low-frequency spark sites (Fig. 2*C* and *D*). However, even at low-frequency sites the spark rate recovered substantially after about 3 s, so it is likely that a lower probability of triggering of fully recovered sites may also be responsible for the long mean intervals of 10 s or greater between spontaneous sparks. A mechanism for this increased probability is suggested by observations that prolonged increases in spark frequency were accompanied by small, localized elevations of basal Ca^{2+} fluorescence (Fig. 4*B* and *C*). Thus, a localized Ca^{2+} leak, either from the SR or the sarcolemma, might promote more frequent sparking. Elevated cytosolic free $[Ca^{2+}]$ might act directly to promote opening of RyR through CICR, or cause a greater local filling of Ca^{2+} stores. (We note that F/F_0 would not reveal elevated cytosolic free $[Ca^{2+}]$ at a frequent site if the $[Ca^{2+}]_i$ was elevated from the beginning of the recording period, and did not change throughout.)

The significance, if any, of 'frequent' spark sites for cell functioning is presently unclear. It might be expected that they would tend to act as foci for initiation of Ca^{2+} waves (cf. Cheng *et al.* 1996), but that could not be substantiated by our data. Whatever, the existence of sites generating frequent sparks provides a useful means for detailed study of sparks, allowing recording of hundreds of repeated events at a single site for statistical analysis or averaging to provide better temporal and spatial resolution.

Different from the 'normal', transient and discrete sparks, some sites also displayed flurries of overlapping sparks or bursts of apparently sustained Ca^{2+} release persisting for hundreds of milliseconds or a few seconds. This behaviour resembles the ryanodine-modified sparks previously described by Cheng *et al.* (1993); except that our results were obtained without pharmacological intervention, suggesting that release sites may spontaneously enter a similar state under physiological conditions. Reconstituted cardiac calcium-release channels display modal changes in gating kinetics, with episodic cycling over several seconds between periods of zero, low or high open probability (Zahradnikova & Zahradnik, 1995). Transitions of channel activity to the high open probability state thus offer an attractive

explanation for the sustained bursts of local Ca^{2+} release – although the relationship between individual channel openings and sparks remains unclear. Cheng *et al.* (1993) interpreted normal and prolonged sparks to arise, respectively, through opening of individual channels to a transitory high conductance state or a prolonged low conductance state. It now appears that sparks reflect the concerted opening of several tightly clustered channels (Lipp & Niggli, 1996; Parker *et al.* 1996; Blatter, Huser & Rios, 1997). In this case, Ca^{2+} flux through a single highly active channel might entrain neighbouring channels to show similar opening behaviour. In fact, we observed coupled bursting behaviour between release sites 1 μm apart (transversely), so it would not be surprising if RyR spaced only nanometers from one another could also 'driven' by a highly active channel within the cluster.

Finally, the long-term changes in spark frequency and patterns of activity from sparks to bursts indicate that properties of release units are not fixed, but may be subject to change by either intrinsic mechanisms (e.g. different modes of RyR channel gating) or extrinsic cellular factors. Modulation of RyR and alteration of Ca^{2+} sparks by the immunophilin FK506-binding protein has been shown recently (Xiao, Valdiva, Bogdanov, Valdivia, Lakatta & Cheng, 1997). Thus, it is possible that excitation–contraction coupling in the heart may be regulated not only by varying the numbers of stereotyped sparks evoked by entry of Ca^{2+} though L-type Ca^{2+} channels, but also by controlling the properties of the elementary Ca^{2+} release units.

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