Characteristics of Prolonged Ca\textsuperscript{2+} Release Events Associated With the Nuclei in Adult Cardiac Myocytes

Zhaokang Yang, Derek S. Steele

Abstract—Confocal microscopy was used to study the properties of nuclear Ca\textsuperscript{2+} regulation in adult ventricular myocytes. Prolonged nuclear Ca\textsuperscript{2+} release (PNCR) events were identified in both intact and permeabilized rat myocytes. PNCR occurred spontaneously and was restricted to localized regions at the ends of the elongated nuclei. Typically, PNCR took the form of a rapid rise in [Ca\textsuperscript{2+}] followed by a maintained plateau. The mean duration of PNCR (1.78±0.19 seconds) was markedly greater than the half decay time for cytosolic Ca\textsuperscript{2+} sparks (31.2±0.56 ms) obtained under the same conditions. The PNCR width at half maximum amplitude (5.0±0.2 μm) was also significantly greater than that of cytosolic Ca\textsuperscript{2+} sparks (2.6±0.05 μm) obtained under the same conditions. Experiments involving the use of syto-11 to accurately locate the nuclei demonstrated that PNCR originates from the nuclear envelope or a closely associated structure. The spatial spread of PNCR was asymmetrical, with greater diffusion of Ca\textsuperscript{2+} toward the center of the nucleus than the cytosol. Both PNCR and Ca\textsuperscript{2+} sparks were abolished by interventions that deplete SR Ca\textsuperscript{2+} stores or inhibit RYR activation. Experiments on intact, electrically stimulated cells revealed that diffusion of Ca\textsuperscript{2+} from the ends of the nucleus toward the center is a prominent feature of the nucleoplasmic Ca\textsuperscript{2+} transient. The possibility that recruitment of Ca\textsuperscript{2+} release sites involved in PNCR might influence the temporal and spatial characteristics of the nucleoplasmic [Ca\textsuperscript{2+}] transient is considered. (Circ Res. 2005;96:82-90.)

Key Words: nucleus ■ sarcoplasmic reticulum ■ Ca\textsuperscript{2+} release ■ sparks

The [Ca\textsuperscript{2+}] within the nucleus regulates a number of important functions including gene transcription and expression, the cell cycle, and apoptosis.1 The double membrane of the nuclear envelope (NE) is spanned by nuclear pore complexes, which allow the passage of solutes and macromolecules <40 kDa. Therefore, nuclear [Ca\textsuperscript{2+}] is at least partly dictated by passive diffusion: global cytosolic [Ca\textsuperscript{2+}] transients or localized Ca\textsuperscript{2+} release events are typically followed by nuclear [Ca\textsuperscript{2+}] transients, which occur with a delay reflecting diffusion of Ca\textsuperscript{2+} across the NE.2 However, there is also evidence that Ca\textsuperscript{2+} can be actively accumulated into the lumen of the NE and then released via Ca\textsuperscript{2+} channels (ryanodine or IP\textsubscript{3} receptors), resulting in a transient rise in nuclear [Ca\textsuperscript{2+}].3–6

The relative influence of these potential sources of nuclear Ca\textsuperscript{2+} varies widely among cell types: in myocardial cells, differing patterns of nuclear Ca\textsuperscript{2+} regulation have been reported in embryonic, neonatal, and adult myocytes. In embryonic chick myocytes, nuclear Ca\textsuperscript{2+} transients can occur without a preceding rise in cytosolic [Ca\textsuperscript{2+}], suggesting that Ca\textsuperscript{2+} may be released from the NE.7 Neonatal cardiac myocytes exhibited both nuclear Ca\textsuperscript{2+} transients and high-frequency Ca\textsuperscript{2+} sparks in the vicinity of the nucleus.8 This study also demonstrated that adult myocytes placed in tissue culture dedifferentiate and develop a pattern of Ca\textsuperscript{2+} release more typical of neonatal cells. Hence, high-frequency perinuclear Ca\textsuperscript{2+} sparks and nuclear Ca\textsuperscript{2+} transients appear characteristic of immature myocardial cells. In part, this may reflect a greater influence of the IP\textsubscript{3} signaling pathway in neonatal or cultured cardiac cells.8 In adult ventricular myocytes, Ca\textsuperscript{2+} sparks arise predominantly at junctional regions of the SR9 and IP\textsubscript{3} receptors are expressed at low levels.10 Furthermore, studies on adult ventricular cells have generally concluded that nucleoplasmic [Ca\textsuperscript{2+}] rises passively because of diffusion from the cytosol, without any contribution from NE derived Ca\textsuperscript{2+}.2,11–13 However, two features of nuclear Ca\textsuperscript{2+} regulation in adult myocytes have yet to be explained. First, the nuclear Ca\textsuperscript{2+} transient lasts significantly longer than the cytosolic Ca\textsuperscript{2+} transient.2,11 Second, the rise in [Ca\textsuperscript{2+}] within the nucleus is biphasic,13 suggesting the possible involvement of a secondary active process.

In the present study, confocal microscopy was used to investigate the properties of nuclear Ca\textsuperscript{2+} regulation in adult ventricular myocytes. Evidence is provided of long-lasting Ca\textsuperscript{2+} release events, which occur spontaneously in localized regions at the ends of the elongated nuclei in both permeabilized and intact cells. The properties of these Ca\textsuperscript{2+} release events and their possible influence on the spatial and temporal properties of the nuclear [Ca\textsuperscript{2+}] transient in intact cells are considered.
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Ca\textsuperscript{2+} markedly inhibits the contractile response with minimal effect on SR movement artifacts associated with contraction. This level of BDM 5 mmol/L 2,3-butanedione monoxime (BDM) was added to prevent exposure of intact cells to 1 mmol/L BODIPY FL C5-ceramide (Molecular Probes), was used as a fluorescent marker of the cell nucleus (for further details see the Expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org).

**Experiments on Permeabilized Cells**

The isolated cells were permeabilized by exposure to saponin (10 μg/mL) in a mock intracellular solution for 6-minute, before centrifugation and resuspension. The intracellular solution contained (mmol/L) 100 KCl, 25 HEPES, 0.36 EGTA, 10 phosphocreatine, 5 ATP, and fluo-3 acid (5 μmol/L); pH 7.0, 20°C to 22°C. The free [Ca\textsuperscript{2+}] and [Mg\textsuperscript{2+}] were adjusted to 200 nmol/L and 1 mmol/L, respectively, by addition of CaCl\textsubscript{2} and MgCl\textsubscript{2}. In most experiments on skinned cells, 5 mmol/L azide was present to inhibit mitochondrial activity. However, azide had no apparent influence on the phenomena reported in the present study. Syto-11 (Molecular Probes), was used as a fluorescent marker of the cell nucleus (for further details see the Expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org).

**Confocal Microscopy**

The experimental chamber was placed on the stage of a Nikon Diaphot Eclipse TE2000 inverted microscope, and the cells were viewed using a 60× water immersion lens (Plan Apo, NA 1.2). A confocal laser-scanning unit (Bio-Rad, Microradiance 2000) was attached to the side port of the microscope. The dyes were excited at 488 nm and emitted fluorescence was measured at >515 nm. Image processing and analysis were done using IDL (Research Systems Inc) and Laserpix (Bio-Rad) and ImageJ (http://rsb.info.nih.gov/ij/) software. Further information regarding the optical properties of the confocal system and image analysis methods are published elsewhere.

**Materials and Methods**

Adult Wistar rats (220 to 250 g; University of Leeds, Leeds, UK) were killed in accordance with the UK Home Office Guidance on the Operation of Animals (Scientific Procedures) Act of 1986. Ventricular myocytes were isolated by collagenase digestion as described previously.14

**Experiments on Intact Cells**

Intact myocytes were perfused with solutions containing (mmol/L) 113 NaCl, 5.4 KCl, 1 MgCl\textsubscript{2}, 1.0 CaCl\textsubscript{2}, 0.37 Na\textsubscript{2}HPO\textsubscript{4}, 5.5 glucose, and 5 HEPES; 20°C to 22°C, pH 7.1. In some experiments, 5 mmol/L 2,3-butanedione monoxime (BDM) was added to prevent movement artifacts associated with contraction. This level of BDM markedly inhibits the contractile response with minimal effect on SR Ca\textsuperscript{2+} release.14 However, the properties of the Ca\textsuperscript{2+} release events reported in the present study appeared similar in the presence or absence of BDM. Changes in cytosolic Ca\textsuperscript{2+} were detected by loading the myocytes with fluo-3AM (5 μmol/L) for 5 minutes at room temperature (20°C to 22°C) as previously described.14 Identification of Ca\textsuperscript{2+} stored within organelles was achieved by loading the cells with fluo-5N as previously described.14 Briefly, intact cells suspended in a solution containing 10 μmol/L fluo-5N AM (Molecular Probes) for 2 hours at 37°C. A 1-hour period was then allowed for de-esterification before commencing the experiment. In some experiments, the Golgi apparatus was identified by exposure of intact cells to 1 μmol/L BODIPY FL C5-ceramide (Molecular Probes) for 1 minute.

**Identification of Prolonged Ca\textsuperscript{2+} Release Events Associated With Nuclei in Permeabilized Cells**

Figure 1 shows sequential x-y images collected at 0.5 Hz from a permeabilized ventricular myocyte. At this low frame rate, cytosolic Ca\textsuperscript{2+} sparks are captured occasionally and, because of their inherently brief nature, occupy only a single frame. However, frames 5 to 8 show a much more prolonged Ca\textsuperscript{2+} release event lasting at least 1.5 seconds and with a greater spatial spread than typical Ca\textsuperscript{2+} sparks. Further examination of the cell using interference microscopy suggested that the prolonged Ca\textsuperscript{2+} release event occurred close to one end of a nucleus.

To gain more information regarding the temporal and spatial properties of these prolonged nuclear Ca\textsuperscript{2+} release (PNCR) events, further experiments were performed in line scan mode. Most ventricular myocytes had two elongated nuclei oriented along the mid axis of the cell. Figure 2A (top) shows a line scan (x-t) image obtained by positioning the scan line longitudinally through both nuclei of a permeabilized rat ventricular myocyte. In this example, a single PNCR was detected from a region of the cell corresponding to one of the nuclei. Much briefer cytosolic Ca\textsuperscript{2+} sparks are also apparent in the same image. Figure 2A (bottom) shows an x-y image of the same cell after exposure to syto-11, which allows the...
boundary of the nucleoplasm and the NE to be defined. The nucleus on the right of the image is apparent as the region of highest fluorescence and is in sharp focus. The x-y and x-t images have been aligned to show that the intersection of the horizontal scan line (white) and the vertical line passing through the peak of the PNCR (red), coincides with the left edge of the nucleus. Figure 2B shows a surface plot and line profiles derived from the data shown in Figure 2A. The initial rise in [Ca^{2+}] peaked at F/F_o = 2.0 and was followed by a plateau, which lasted 1.4 seconds, before decreasing to cytosolic levels. In contrast, cytosolic Ca^{2+} sparks were much briefer and did not exhibit a plateau.

PNCR was typically associated with a limited diffusion of Ca^{2+}, both toward the center of the nucleus and toward the cytosol (eg, Figure 2A). However, Ca^{2+} diffused further into the nucleoplasm than into the cytosol: Figure 2C (left), shows...
a surface plot of a PNCR event, which has been oriented to show the spatial spread of \( \text{Ca}^{2+} \) into the cytosolic and nuclear compartments. In this example, the asymmetry is particularly apparent, with greater spread into the nucleoplasm. As the plateau declined, the spatial spread also decreased gradually, before \( \text{Ca}^{2+} \) release suddenly terminated. Normalized data illustrating the signal-averaged spatial profiles of PNCR events from 12 cells is also shown (right). As suggested in relation to other perinuclear \( \text{Ca}^{2+} \) release events, the asymmetrical \( \text{Ca}^{2+} \) spread probably reflects differences in the density of \( \text{Ca}^{2+} \) binding sites between the nuclear and cytosolic compartments.  

On average, PNCR was detected with equal frequency at the left and right edges of the nuclei (\( n = 68 \) cells), and in-focus events were consistently located at or near the nuclear boundary, as defined by syto-11 fluorescence. However, PNCR was not detected when the scan line was positioned transversely across the midpoint of each nucleus (not shown), indicating that the events are localized to specific regions at the ends of the elongated nuclei. PNCR was not observed following inhibition of RyR activation with high levels of ryanodine (20 \( \mu \text{mol/L} \), not shown). Similarly, PNCR was not detected after depletion of intracellular \( \text{Ca}^{2+} \) stores by introduction of 10 \( \mu \text{mol/L} \) caffeine (to activate RyR) or 20 \( \mu \text{mol/L} \) cyclopiazonic acid (to inhibit SERCA).

In contrast to results obtained on some other cell types,  

Further Properties of Long-Lasting \( \text{Ca}^{2+} \) Release Events

On average, PNCR was detected at a frequency of 2.5±0.3 (\( n = 33 \)) events per minute, per nucleus. However, in some cells, PNCR occurred at a higher frequency from the same focal point (Figure 3A). Typically, in-focus PNCR events exhibited a rapid rising phase, which peaked at \( F/F_0 = 2.0 \) (see next section for cumulative data). However, in approximately 5% of cells, rapid changes between full and half maximum amplitude were observed (Figure 3B). The longest PNCR event detected lasted \( \approx 8 \) seconds, indicating the presence of a substantial \( \text{Ca}^{2+} \) pool (Figure 3C). Similar prolonged events were observed in intact cells loaded with fluo-3AM (Figure 3D).

Cumulative Data

Histograms illustrating the temporal and spatial properties of PNCR and cytosolic \( \text{Ca}^{2+} \) sparks are given in Figure 4. There was no significant difference between the mean amplitude of \( \text{Ca}^{2+} \) sparks (\( F/F_0 = 2.0 \pm 0.03, n = 361 \)) and PNCR events (\( F/F_0 = 2.0 \pm 0.04, n = 68 \)). The mean duration of PNCR was significantly greater (1.78±0.19 seconds, \( n = 68 \)) than the half decay time for \( \text{Ca}^{2+} \) sparks (31.2±0.56 ms, \( n = 361; P < 0.01 \)). The PNCR half width at the point of maximum amplitude (5.0±0.2 \( \mu \text{m} \), \( n = 68 \)) was also significantly greater than that of typical \( \text{Ca}^{2+} \) sparks (2.6±0.05 \( \mu \text{m} \), \( n = 311; P < 0.01 \)). Whereas the amplitude distribution of \( \text{Ca}^{2+} \) sparks exhibited a typical Gaussian relationship (as in most previous studies), PNCR events appeared more bell shaped, with a higher proportion of low amplitude events being detected. This may reflect the fact that low amplitude events are easier to discriminate from background noise when they last seconds as opposed to milliseconds.

As shown in Figure 2, PNCR typically exhibited a rapid rising phase followed by a slow monotonic decrease, before the final sudden decline in amplitude on termination of \( \text{Ca}^{2+} \) release. At the point of release termination, the mean PNCR amplitude was 57.3±2.4% (\( n = 68 \)) of the initial peak. The spatial spread of PNCR also decreased as the amplitude declined (eg, Figure 2C). On average, PNCR width (FWHM) decreased by 20.1±2.5% (\( n = 68 \)) at the point of release termination.

Changes in Nuclear \([\text{Ca}^{2+}]_n\) in Intact Cells During Electrical Stimulation

Further experiments were performed on intact myocytes to establish whether the inward diffusion of \( \text{Ca}^{2+} \) from the ends of the nuclei plays a role in the beat-to-beat regulation of nuclear \([\text{Ca}^{2+}]_n\). Figure 5A shows an \( x-t \) image of an intact ventricular myocyte during an electrically stimulated response. The scan line was positioned longitudinally through one of the nuclei. On stimulation, the rapid rise in \([\text{Ca}^{2+}]_n\) resulted in a horizontal line across cytosolic regions of the line scan image. The \([\text{Ca}^{2+}]_n\) also increased within the nucleus, but with a delay reflecting diffusion of \( \text{Ca}^{2+} \) across the NE. As reported previously, 2,11 the rise in \([\text{Ca}^{2+}]_n\) within the nucleus was markedly prolonged relative to the cytosolic \( \text{Ca}^{2+} \) transient. However, one feature not highlighted in earlier studies is the apparent ‘V’ in the fluorescence signal, joining
the ends of the nucleus. This is consistent with diffusion of Ca\(^{2+}\) from the ends of the organelle toward the center. The expanded panel (bottom) shows the same image, with the nucleoplasmic region blanked and the time scale compressed. The arrows indicate regions of high localized Ca\(^{2+}\), which appear to spread outwards into the cytosol before slowly receding.

Selected line profiles (Figure 5B) show the change in [Ca\(^{2+}\)] within the cytosol (i), just inside the nucleus (ii), and at the center of the nucleus (iii). The rise in [Ca\(^{2+}\)] just inside the NE was slightly delayed compared with the cytosolic Ca\(^{2+}\) transient and was typically monotonic. However, the rise in [Ca\(^{2+}\)] at the center of the nucleus was markedly slower and the maximum level lower than at the ends of the nucleus. In addition, the line profile from the center of the nucleus exhibited a secondary rise in [Ca\(^{2+}\)] (red arrow). Comparison of the line profile and the line scan, suggest that the secondary rise in Ca\(^{2+}\) reflects the influence of Ca\(^{2+}\) diffusing inwards from the ends of the nucleus. Similar results were obtained in 11 other preparations.

Cumulative data illustrating the time courses of the rising and falling phases in regions (i), (ii), and (iii) is given in Figure 5C. The mean propagation rate of Ca\(^{2+}\) from the ends of the nucleus toward the center was 49.8±3.7 \(\mu\)m/sec (n=12). The time to peak (measured at 90% max) of the cytosolic Ca\(^{2+}\) transient (i), and regions (ii) and (iii) of the nucleus was 20.4±1.8 ms, 46.5±5.4 ms, and 157.8±15.1 ms (n=12), respectively. The corresponding descending phases declined with half times of 398.3±49.3 ms, 589.2±71.8, and 598.3±82 ms (n=12).

The role of Ca\(^{2+}\) diffusion from the ends of the nucleus toward the center is further highlighted by sequential x-y images obtained from another cell after electrical stimulation (Figure 5D). The selected frames reveal localized regions of high fluorescence at the ends of the nucleus. These regions broaden, producing a characteristic “dumbbell” appearance, before spreading inwards toward the center of the nucleus. Similar results were obtained in 6 other preparations.

**Location of Intracellular Ca\(^{2+}\) Storage Sites in Intact Cells**

The distribution of stored intracellular Ca\(^{2+}\) was investigated by exposure of intact cardiac cells to fluo-5N AM, under conditions that favor compartmentalization of dye in the organelles. Figure 6A shows an x-y image of fluo-5N fluorescence centered on one of the two nuclei. As previously
reported, a characteristic sarcomeric pattern of staining is present, which corresponds to dye trapped within the lumen of the SR.16 The NE is also visible because of Ca²⁺ stored within the perinuclear space. However, another consistent feature was the presence of localized areas of high fluorescence at the ends of the nuclei, suggesting regions of high organelle Ca²⁺.

Unlike previous reports in rabbit myocytes,16 electrical stimulation did not result in a decrease in Fluo-5N fluorescence (not shown). This might reflect (1) a higher than expected Ca²⁺ affinity of the de-esterified dye complexes and/or (2) a higher luminal [Ca²⁺] within the SR of rat compared with rabbit myocytes. However, a pronounced reduction in both sarcomeric and perinuclear fluorescence did occur after maximal depletion of the SR Ca²⁺ stores by inhibition of SERCA (with CPA) followed by RYR activation by application of 10 mmol/L caffeine (Figure 6B). This effect was present, but less pronounced with caffeine alone (not shown), which may reflect the fact that the SR can retain Ca²⁺ in the presence of caffeine, when SERCA is active.20

The bright region at the ends of the nuclei might reflect Ca²⁺ stored in the NE or adjoining regions of the SR. However, ultrastructural studies suggest that the Golgi appa-
The mitochondria are an unlikely source of Ca^{2+} because spontaneous PNCR was observed in the presence or absence of mitochondrial inhibitors. It is less easy to draw firm conclusions about the involvement of the NE, SR, or GA. Both the SR and the NE membranes of cardiac cells contain SERCA2a and RYR2. In addition, the lumen of the SR/ER is continuous with the lumen of the NE. Therefore, any factor affecting the Ca^{2+} content of the SR will affect the NE and vice versa. Recent work has also highlighted the possible role of the GA as a functional Ca^{2+} store: the GA has two forms of Ca^{2+} pump (SERCA and a P-type SPCA) and in some cells types IP3 and ryanodine (RYR2) receptors are also associated with the GA membrane.

In the present study, PNCR was abolished by inhibition of SERCA (with CPA), or drugs, which activate or inhibit RYR function (caffeine or ryanodine). Comparison of the images obtained using fluo-5N loading (Figure 4A) and C5-ceramide (Figure 6B) suggest that the regions of high [Ca^{2+}] at the ends of the nuclei may partly reflect Ca^{2+} localized in the GA. However, exposure to caffeine and thapsigargin appeared to deplete Ca^{2+} within the SR, NE, and the regions at the nuclear poles (Figure 6A). Therefore, the shared components of the Ca^{2+} regulatory systems may preclude pharmacological differentiation between these potential sources of Ca^{2+}. Despite this limitation, several features of PNCR are more consistent with the involvement of the SR or NE than the GA. First, PNCR was abolished by high levels of ryanodine (20 μmol/L), but was not induced or modified by IP3. This suggests that PNCR is likely to involve Ca^{2+} efflux via RYRs. Second, activation of Ca^{2+} release from the GA in response to RYR agonists has not been demonstrated in any cell type, suggesting that the NE or adjoining regions of the SR are more probable sources of Ca^{2+}. Whatever its origin, the functional importance of PNCR primarily reflects its location and influence on nuclear Ca^{2+}.

### Temporal Properties of Ca^{2+} Sparks and Prolonged Ca^{2+} Release Events

A number of mechanisms have been proposed to explain the temporal properties of Ca^{2+} sparks and long-lasting Ca^{2+} release events observed after pharmacological modification of RYR function. These include (1) Ca^{2+}-dependent channel inactivation or adaptation, (2) partial depletion of luminal Ca^{2+} or (3) stochastic attrition. Models involving only one of these potential mechanisms have generally failed to describe accurately the properties of Ca^{2+} sparks. Moreover, such models are limited by uncertainties over the single RYR Ca^{2+} flux and the number of channels activated during a Ca^{2+} spark or a prolonged Ca^{2+} release event. If sparks reflect the activation of tens or perhaps hundreds of RYRs, simple explanations based on stochastic attrition (simultaneous stochastic closing of RYRs within a cluster) can be excluded. However, recent models incorporating evidence that RYRs can be functionally coupled, with other features such as Ca^{2+} depletion of the SR, have proved more successful.

It has been shown that pharmacological modification of the RyR can result in long-lasting Ca^{2+} release events, with similar properties to PNCR, eg, Ca^{2+} sparks with prolonged “embers” can be induced in cardiac cells by the drugs FK-506.
or rapamycin, which dissociate FKBP from RYR. In this case, it was suggested that Ca\(^{2+}\) release is prolonged because of reduced coupling between RYRs, which impairs the ability of closed channels to facilitate the inactivation of others. However, long-lasting events can also be induced by other substances including ryanoids because of induction of long-lasting subconductance states. The fact that modification of RYR gating can produce such long-lasting events confirms that complete depletion of local Ca\(^{2+}\) stores cannot explain the normally brief nature of Ca\(^{2+}\) sparks. However, partial depletion may contribute to spark inactivation by reducing the open probability of the RyR.

The present study shows that long-lasting Ca\(^{2+}\) release events can occur from localized regions at the ends of the nuclei in the absence of pharmacological agents. The prolonged nature of these events might reflect local structural characteristics, e.g., the number of RYRs per cluster, the organization/packing of RYRs, or the degree of coupling conferred by FKBP. Other factors, such as the local phosphorylation levels of phospholamban or RyR may also influence channel inactivation characteristics, thereby prolonging Ca\(^{2+}\) release. Interestingly, "hotspots" of colocalized PKA and calcineurin have been reported to exist at the ends of the nuclei in adult cardiac myocytes (see Figure 7 in Santana et al).

Recent models suggest that a maintained Ca\(^{2+}\) flux of 10% to 20% of that associated with a Ca\(^{2+}\) spark is sufficient to produce a prolonged Ca\(^{2+}\) release event, similar to that shown in Figure 2. The rapid transitions between full and half maximal amplitude that occur in some PNCR events (Figure 3B) could be explained by a number of possible mechanisms, e.g., if PNCR involves the maintained activation of a single RyR, then a 50% decrease in amplitude might reflect the adoption of a subconductance state. However, if many RYRs are active during PNCR then such a decrease in amplitude could be explained if tight coupling between active RyRs enables a simultaneous transition to a subconductance state.

**Physiological Relevance**

The spontaneous PNCR events observed in skinned cells (Figure 1) are unlikely to have a role under physiological conditions. This is because the mean duration of PNCR is substantially greater than the cardiac cycle and each rise in [Ca\(^{2+}\)] during systole would be expected to disrupt or entrain spontaneous PNCR. However, spontaneous PNCR may play a role in controlling Ca\(^{2+}\)-dependent nuclear processes under pathological conditions, when the cell becomes quiescent and Ca\(^{2+}\) regulation is impaired, e.g., during ischemia or reperfusion.

One important consideration is whether the Ca\(^{2+}\) release sites, which underlie spontaneous PNCR in skinned and intact cells, are activated during the electrically stimulated Ca\(^{2+}\) transient. Evidence of Ca\(^{2+}\) diffusion from the ends of the nuclei toward the center is consistent with this possibility (Figure 5A). Furthermore, as with spontaneous PNCR (Figure 2), there is evidence of a prolonged outward diffusion of Ca\(^{2+}\) from a region at the edge of the nucleus toward the cytosol (Figure 5A, bottom). This pattern of Ca\(^{2+}\) release cannot readily be explained by simple inward Ca\(^{2+}\) diffusion from the cytosol to the nucleoplasm and suggests that the Ca\(^{2+}\) release sites, which underlie spontaneous PNCR, are activated during the electrically stimulated Ca\(^{2+}\) transient. PNCR might be triggered via CICR, following each global rise in cytosolic [Ca\(^{2+}\)], or by Ca\(^{2+}\) entry across closely associated t-tubule membranes.

Assuming the Ca\(^{2+}\) release sites, which underlie PNCR are recruited during each beat, then the inherently prolonged nature of the underlying Ca\(^{2+}\) release process may contribute to the long-lasting nucleoplasmic Ca\(^{2+}\) transient (eg, Figure 5A). Such a mechanism could be of considerable importance given that nuclear Ca\(^{2+}\) is involved in the regulation gene transcription and expression. Of particular interest to the cardiac field, recent work has shown that changes in nucleoplasmic Ca\(^{2+}\) plays a central role in the development of hypertrophy.

**Conclusion**

The present study has identified novel long-lasting Ca\(^{2+}\) release events, which occur spontaneously at the ends of the nuclei in both permeabilized and intact myocytes. In intact electrically stimulated cells, the inward diffusion of Ca\(^{2+}\) from the ends of the nuclei is a prominent feature of the nucleoplasmic Ca\(^{2+}\) transient and this appears to explain the biphasic increase in [Ca\(^{2+}\)] at the center of the organelle. The association of PNCR events with the ends of the nuclei suggests that recruitment of the underlying Ca\(^{2+}\) release sites during systole may contribute to the beat-to-beat regulation of nuclear Ca\(^{2+}\).

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**References**


16. Shannon TR, Guo T, Bers DM. Ca\textsuperscript{2+} sparks: local depletions of free [Ca\textsuperscript{2+}] in cardiac sarcoplasmic reticulum during contractions leave substantial Ca\textsuperscript{2+} reserve. *Circ Res.* 2003;93:40–45.


24. Pinton P, Pozzan T, Rizzuto R. The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca\textsuperscript{2+} store, with functional properties distinct from those of the endoplasmic reticulum. *EMBO J.* 1998;17:5298–5308.


27. Bassani JWM, Yuan WL, Bers DM. Fractional SR Ca\textsuperscript{2+} release is regulated by trigger Ca\textsuperscript{2+} and SR Ca\textsuperscript{2+} content in cardiac myocytes. *Am J Physiol.* 1995;37:C1313–C1319.


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Materials and Methods

As in previous studies, syto-11 (Molecular Probes, Leiden, Netherlands), was used as a fluorescent marker of the cell nucleus\(^1\). Syto-11 binds to nucleic acids, which are concentrated in the nucleus, but may also in other cellular compartments including the mitochondria. As the present study required accurate identification of the nucleus, the method was validated by comparing the syto-11 fluorescence pattern with that produced by the lipophilic dye Di-8 ANNEPS (Biotium, USA). Figure 1A shows a fluorescence image obtained from a permeabilized myocyte following 5-minutes exposure to 5 µM Di-8-ANNEPS. Both the membrane of the NE and a regular striated pattern are apparent. The striated pattern is at least partly due to binding of dye to the t-tubules\(^2\), but may also reflect binding to intracellular membranes, including the junctional SR. Figure 1B shows an image taken from the same cell following a further 3-minutes perfusion with a solution containing 250 µM syto-11. In the presence of syto-11, the nucleus appears as the region of brightest fluorescence (Figure 1B). In Figure 1C, the images obtained using Di-8-ANNEPS and syto-11 have been combined to show that the outer edge of the nuclear syto-11 fluorescence pattern accurately defines the boundary between the nucleoplasm and the surrounding NE. However, it was found that syto-11 identified the nucleus more rapidly and clearly than Di-8-ANNEPS. Therefore, in the present study, syto-11 was used routinely to identify the nucleus.

![Di-8 ANNEPS, syto-11, combined](image)

Figure 1 A, Di-8-ANNEPS fluorescence from a permeabilized myocyte indicating dye bound to cell membranes including the nuclear envelope. B, image from the same cell showing nucleoplasmic localisation of the syto-11 fluorescence. C, composite image, illustrating that syto-11 accurately defines the boundary between the nucleoplasm and the NE.
References
