Depressed Ryanodine Receptor Activity Increases Variability and Duration of the Systolic Ca\(^{2+}\) Transient in Rat Ventricular Myocytes


Abstract—Sarcoplasmic reticulum (SR) Ca\(^{2+}\) release, through the ryanodine receptor (RyR), is essential for the systolic Ca\(^{2+}\) transient and thus the cardiac contractile function. The aim of this study was to examine the effects on the spatial organization of the systolic Ca\(^{2+}\) transient of depressing RyR open probability (P\(_o\)) with tetracaine or intracellular acidification. Voltage-clamped, fluo-3–loaded myocytes were studied using confocal microscopy. Depressing RyR P\(_o\) increased the variability of the Ca\(^{2+}\) transient amplitude between different regions of the cell. This variability often produced alternans with a region producing large and small transients alternately. In addition, the raising phase of the Ca\(^{2+}\) transient became biphasic. The initial phase was constant but the second was variable and propagated as a wave through part of the cell. That both phases involved SR Ca\(^{2+}\) release was shown by their reduction by caffeine. Regional [Ca\(^{2+}\)]i alternans was accompanied by a much smaller degree of alternans at the whole cell level. We suggest that, in tetracaine or acidosis, the initial phase of the Ca\(^{2+}\) transient results from Ca\(^{2+}\) release via RyRs directly activated by adjacent L-type Ca\(^{2+}\) channels. At some sites, this will activate neighboring RyRs and a Ca\(^{2+}\) wave will propagate via activation of other RyRs. This work is the first demonstration that decreased RyR P\(_o\) alone can produce disarray of the Ca\(^{2+}\) release process and initiate alternans. (Circ Res. 2002;91:585-593.)

Key Words: calcium ■ ryanodine receptor ■ alternans
solution contained (in mmol/L) NaCl 135, Glucose 11, CaCl$_2$ 1, access resistance was typically about 20 MΩ. The superfusing solution contained (in mmol/L) NaCl 135, Glucose 11, CaCl$_2$ 1, HEPES 10, MgCl$_2$ 1, and KCl 20, NaCl 10, HEPES 10, and MgCl$_2$ 5, titrated to pH 7.2 with KOH and amphotericin-B (final concentration, 240 µg/mL). Final access resistance was typically about 20 MΩ. The superfusing solution contained (in mmol/L) NaCl 135, Glucose 11, CaCl$_2$ 1, HEPES 10, MgCl$_2$ 1, and KCl 4, titrated to pH 7.4 with NaOH. In voltage clamp, 5 mmol/L 4-aminopyridine and 0.1 mmol/L BaCl$_2$ were also added to block outward currents. Tetracaine was added at a final concentration of 50 µmol/L. Acidification of the cytoplasm was induced by exposing the cells to Butyrate (30 mmol/L) in the presence of Dimethyl amiloride (0.02 mmol/L) maintaining extracellular pH constant. All experiments described in detail were performed at room (24°C) temperature; however, similar results were also seen at 37°C (see online data supplement). Where applicable, the data are reported as the mean±SEM of n experiments, significance was tested using paired Student’s t test.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Variability of Ca$^{2+}$ release during systole under control conditions can be seen in Figure 1. The line scans in Figure 1A are from consecutive stimuli under voltage clamp with the laser scanning along the long axis of the cell. The “ragged” appearance of the rise in Ca$^{2+}$ shows individual Ca$^{2+}$ release sites analogous to sparks. Release occurs at some sites earlier than at others. Sites marked by small arrows release early on all stimuli. In contrast, the sites marked by large arrows show early release on only some pulses. In other words, there is variability from pulse to pulse in the response of a given site.1 This is emphasized by the profiles shown in Figure 1B, which show the average intensity of the line scan as a function of distance over the first 20 ms after depolarization. Again, sites that responded to each pulse are indicated by small arrows and variable sites by large arrows. Importantly, it is possible to locate widely separated regions that respond on each pulse; this makes it unlikely that variability at other sites is due to movement of the cell.

Experiments such as that of Figure 1 demonstrate variability over small (<5 µm) regions. In subsequent experiments, we have investigated the effect of altering the properties of the RyR. At first, we used the local anesthetic tetracaine to decrease the RyR P$_{o}$.15 This produces an initial decrease of systolic [Ca$^{2+}$], and contraction due to depressed RyR function before systolic [Ca$^{2+}$], recovers as SR Ca$^{2+}$ content increases.3 The effects of tetracaine on a cell studied under confocal microscopy are shown in Figure 2. The first two panels of Figure 2A show control line scans, similar to those of Figure 1. Subsequent line scans in tetracaine show marked large scale heterogeneity in the amplitude of the increase of [Ca$^{2+}$]. Importantly, regions showing a large increase of [Ca$^{2+}$], (eg, top part of panel d) showed a smaller increase in the next stimulus (e); conversely, the largest increase of [Ca$^{2+}$], in e occurred in a region that experienced little increase of [Ca$^{2+}$], in d. The time course of events is more obvious in Figure 2B. The upper two traces (labeled i and ii) show measurements of [Ca$^{2+}$], in small regions of the cell. Application of tetracaine reduces Ca$^{2+}$ release in both regions but, as the Ca$^{2+}$ transient amplitude recovers, clear alternans develop. Initially, these are in phase in the two regions but, toward the end of the exposure to tetracaine, they are out of phase. The next trace shows global [Ca$^{2+}$], from the whole line scan. When regions i and ii are in phase, the global [Ca$^{2+}$], demonstrates marked alternans. In contrast, when the two regions are out of phase the alternation of the whole line scan is less obvious although still present. The bottom trace is a measurement of contraction (see Materials and Methods). This is qualitatively similar to the global [Ca$^{2+}$], although it appears that the variation in amplitude of contraction is fractionally larger than that of [Ca$^{2+}$], perhaps reflecting the steepness of the relationship between [Ca$^{2+}$], and contraction. The results of this experiment demonstrate that the degree of alternans in regions of the cell can be much larger than that seen at the level of the whole cell. Such regional alternation in the Ca$^{2+}$ transient amplitude in tetracaine was seen in 16 out of 20 cells. Although in previous work studying the effects of tetracaine, we have not commented on the presence of alternans, there are examples in, eg, Figures 1 and 2 of Overend et al.,3 of increased variability and alternans in contraction and systolic Ca$^{2+}$.
Another important finding (particularly evident in Figure 2Ae) is the spread of the increase of \([\text{Ca}^{2+}]_i\) as a wave away from the initiating site (see region below the dashed line). This phenomenon will be addressed in Figure 3. However, Figure 2C shows that as a result of the wave both time to peak and decay of the global \([\text{Ca}^{2+}]_i\) transient are increased. This, in turn, causes the kinetics of contraction to be slowed. On average time to peak of the global \([\text{Ca}^{2+}]_i\) transient is increased in tetracaine from 107±9 to 126±33 ms (P<0.04, n=7) and the rate constant of decay decreased from 4.0±0.6 to 3.0±0.7 s\(^{-1}\) (P<0.002, n=7), ie, the transient is slower to rise and decay.

The wave-like nature of release is also evident in Figure 3 (obtained in tetracaine). The specimen records in Figure 3B were measured as indicated in the line scan in Figure 3A. It can be seen that transients a, b, and c rise to an initial level before a secondary increase of \([\text{Ca}^{2+}]_i\). This secondary increase takes place progressively later from a to c as the wave propagates. The transient labeled d did not show this secondary increase because the wave did not propagate into this region. The relative kinetics of the various transients are best seen in the left panel of Figure 3C. This shows that the amplitude of the initial increase of \([\text{Ca}^{2+}]_i\) is similar in all the transients (transient b is omitted for clarity) although the rate of rise of transient a is lower (cf, Figure 1). This initial increase is also uniform from stimulus to stimulus (not shown). Such biphasic \([\text{Ca}^{2+}]_i\) transients (with the second phase more variable) were seen in 17 of 20 cells studied. The delayed release during the wave will obviously increase the time for the global \([\text{Ca}^{2+}]_i\) transient to reach a peak. The right panel of Figure 3C also illustrates how the wave may slow the falling phase of the global \([\text{Ca}^{2+}]_i\) transient. The noise-free (black) trace is global \([\text{Ca}^{2+}]_i\) from line scan A, the second phase of \([\text{Ca}^{2+}]_i\) release in transient c is still rising when the overall \([\text{Ca}^{2+}]_i\) signal is falling. Release of \([\text{Ca}^{2+}]_i\) taking place at this time must slow the fall of global \([\text{Ca}^{2+}]_i\). It should be noted that, for experimental convenience, the experiments described were performed at room temperature. However, qualitatively similar effects of tetracaine were also observed at 37°C (see online Figures 1 and 2, available in the online data supplement).
The idea behind using tetracaine to enhance release variability rests on its ability to decrease RyR $P_o$. Acidification also lowers RyR $P_o$ and produces similar effects to tetracaine on the whole-cell Ca$^{2+}$ transient. Acidic pH also favors the appearance of alternans. We, therefore, investigated whether acidifying the cytoplasm (maintaining extracellular pH constant) would increase Ca$^{2+}$ release variability. The control line scans in Figure 4 show very little variability of release but application of 30 mmol/L butyrate (lowering intracellular pH by approximately 0.3 units) produced regional alternans very similar to those in tetracaine. Similar phenomena were seen in 5 of 7 cells examined. The top trace in Figure 4B shows that fluorescence measured from the region between the horizontal white lines shows alternation. The global fluorescence shows less alternation. Therefore, the three characteristics of the response to tetracaine are also produced by acidification: local alternans; Ca$^{2+}$ release in two distinct phases (more clearly seen in Figure 5); the second phase of release propagates as a wave.

In both tetracaine and butyrate, the Ca$^{2+}$ transient has an initial rise followed by a delayed further increase. The initial increase of [Ca$^{2+}$], might be as follows: (1) Ca$^{2+}$ entry on L-type Ca$^{2+}$ current or (2) an initial phase of SR Ca$^{2+}$ release. We have distinguished between these by removing Ca$^{2+}$ release by exposure to caffeine (10 mmol/L). Line scan a of Figure 5 was obtained in 30 mmol/L butyrate. Two transients are shown below, taken from the points indicated. One shows only the initial increase of [Ca$^{2+}$], whereas in the other, after the initial increase, there is a second increase of [Ca$^{2+}$]. The other two line scans were obtained in the presence of caffeine (when [Ca$^{2+}$], had decayed after the initial release, b) and shortly (c, 5 seconds) after removing caffeine. The line scans and specimen records in Figure 5B show that the rise of [Ca$^{2+}$] in caffeine is significantly smaller in amplitude and much more slowly rising than is the initial phase of the Ca$^{2+}$ transient in the absence of caffeine (the traces in caffeine are averages over the whole line scan); similar results were seen in another 3 cells. We therefore conclude that the initial and delayed phases of the Ca$^{2+}$ transient are largely due to SR Ca$^{2+}$ release.

In Figure 2, we saw that the degree of alternans or variability observed at a subcellular level is greater than that at even the level of the whole cell. In Figure 6, we compare this subcellular variability with that observed at the level of the whole cell. Clear regional variability of release is evident in regions in the line scans of Figure 6A (tetracaine is present throughout). The top trace of Figure 6B shows the global fluorescence relationship obtained from the whole line.
The dashed line shows the small degree of variability in the amplitude of this transient. Of course, a single line scan measures changes of $[\text{Ca}^{2+}]_i$ in only a very small fraction of the cell volume. An index of the mean rise of $[\text{Ca}^{2+}]_i$ in the whole cell can be obtained from the Na$^+$-Ca$^{2+}$ exchange (NCX) tail current ($I_{\text{NCX}}$) on repolarization. This is best seen from the integrated current records in the bottom panel of Figure 6B. This shows the calculated Ca$^{2+}$ entry (L-type Ca$^{2+}$

![Figure 4.](image-url) **Figure 4.** Effects of intracellular acidification with butyrate. A, Line scans. Left two line scans were obtained in control and the other beginning after 55-second exposure to butyrate (30 mmol/L). B, Specimen data from the line scans in A. Top record shows fluorescence measurements in the local region shown on A, whereas the lower region is the average of the whole scanned line.

![Figure 5.](image-url) **Figure 5.** Both the initial and delayed phases of the Ca$^{2+}$ transient appear to result from SR Ca$^{2+}$ release. A, Lines cans. a was obtained in the presence of butyrate (30 mmol/L), b after 30-second exposure to 10 mmol/L caffeine, and c 5 seconds after removing caffeine. In all panels, a 100-ms duration depolarizing pulse began 100 ms after the start of the line scan. B, Specimen records. Left two records (i and ii) were obtained at the positions indicated on a. Next two traces show average of all the pixels in b and c. Bottom record shows i and ii and the response to stimulation in caffeine superimposed.
current integral) on each pulse. For the 6 pulses shown, this Ca\(^{2+}\) entry is constant from pulse to pulse (4.07 ± 0.02 μmol/L; maximum difference between consecutive pulses of 0.13 μmol/L). In contrast, Ca\(^{2+}\) efflux on NCX (shown as downward deflections) is larger on pulses b, d, and f. The maximum difference of Ca\(^{2+}\) efflux between consecutive pulses was much greater than that of influx, ie, 1.2 μmol/L. In other words there is alternation in the whole-cell Ca\(^{2+}\) transient amplitude, showing that a small degree of alternation/variability at the whole-cell level may be due to a much larger degree at the subcellular level. Importantly, the degree of alternation at the whole-cell level (as measured from the I\(_{\text{NCX}}\) integral) is similar to that of the average of the line scan. The alternans present in tetracaine and low pH might be sustained to some extent if the large transient depletes the SR for the subsequent release. A smaller release would, in turn, allow extra Ca\(^{2+}\) accumulation for the following cycle. If such depletion/accumulation cycles are taking place, we should be able to measure the Ca\(^{2+}\) fluxes across the surface membrane responsible under voltage-clamp conditions. Figure 6 shows that small changes of net cellular Ca\(^{2+}\) flux do occur. However, because alternation is most prominent at a subcellular level, the important question is whether larger changes of SR Ca\(^{2+}\) content are expected at the subcellular level? In order to test this, we need to know the amount of Ca\(^{2+}\) efflux a local transient would activate. The relationship between the I\(_{\text{NCX}}\) and the Ca\(^{2+}\) transient averaged over the whole line scan tells us how much current (at the whole-cell level) a given size transient activates. We can express this current as a change of total cell Ca\(^{2+}\) per unit volume. We assume that a local change of [Ca\(^{2+}\)] will result in an equivalent change of local total Ca\(^{2+}\) (in other words, we are assuming that neighboring regions of the SR do not communicate with the region in which the local transient occurs). Given the area occupied by the local transients, this seems reasonable. We have done this calculation for the data in Figure 7A. Each line scan is shown with its current record and the local Ca\(^{2+}\) transient at the point indicated. The bottom panel shows the Ca\(^{2+}\) fluxes. The black lines were obtained by integrating the experimentally measured current traces and therefore represent the flux balance of the whole cell. The red lines were calculated using the estimated I\(_{\text{NCX}}\) as described above. As noted for the cell shown in Figure 6, local alternation of release has little effect on overall Ca\(^{2+}\) transient amplitude, showing that a small degree of alternation/variability at the whole-cell level may be due to a much larger degree at the subcellular level. Importantly, the degree of alternation at the whole-cell level (as measured from the I\(_{\text{NCX}}\) integral) is similar to that of the average of the line scan.

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Ca^2+ measurements and thus estimations of local efflux, this is unlikely to significantly alter the conclusion that little loss of Ca^2+ takes place locally.

An expanded Results section can be found in the online data supplement available at http://www.circresaha.org.

**Discussion**

The data in this article show that decreasing the RyR P_o (by tetracaine or acidification) increases the degree of spatial and temporal desynchronization of SR Ca^2+ release. This has two important consequences: (1) in a given region of the cell, SR Ca^2+ release propagates as a wave and its amplitude can alternate from stimulus to stimulus. The degree of alternation observed at the subcellular level can be much larger than that observed at the level of the whole cell. (2) There is slowing in the apparent rate of rise and decay of the Ca^2+ transient.

Under control conditions, there is some regional variation in the time of onset of the Ca^2+ transient. This variation occurs over small distances (of the order of up to 5 μm; Figure 1) and disappears within 40 to 50 ms of the start of a
Regional Release Sites Produce Biphasic Ca\textsuperscript{2+} Release and Miniwaves

In the presence of tetracaine, Ca\textsuperscript{2+} release occurs in a biphasic manner. An initial Ca\textsuperscript{2+} release is followed after a variable delay by a second phase. That both phases are due to SR release (rather than to Ca\textsuperscript{2+} entry into the cell) is shown by their sensitivity to caffeine (Figure 5).

The first phase is reasonably constant in both amplitude and time course (Figure 3). In contrast, the second shows both temporal and spatial heterogeneity and underlies the alternation of the amplitude of the Ca\textsuperscript{2+} transient. On a given pulse, the second component spreads as a wave through a limited region of the cell.

It is important to consider why the secondary phase of release occurs in only a limited number of regions and spreads as a wave. One explanation is based on the idea of two groups of RyRs: (1) those “coupled” to L-type channels and (2) “uncoupled” clusters not associated with L-type channels (analogous to “orphan receptors”\textsuperscript{21}) or for which the associated L-type channel has not opened. We assume that, under control conditions, Ca\textsuperscript{2+} entry on L-type channels activates Ca\textsuperscript{2+} release from coupled RyRs that can activate release from neighboring, uncoupled RyRs. This whole process may occur sufficiently quickly as to appear experimentally as one phase. In the presence of tetracaine, the RyRs P\textsubscript{o} is decreased.\textsuperscript{15} This may not be sufficient to stop the coupled RyR opening in response to the L-type Ca\textsuperscript{2+} entry, but it may prevent coupled release from stimulating uncoupled RyRs, barring a few sites where, e.g., inactivation of release is low or SR content is high. Ca\textsuperscript{2+} release can then spread from these sites as a wave and activate other RyRs. The effectiveness as a trigger of the wave may be enhanced as it arrives before the initial phase of release has recovered (Figure 3). The second phase of release may be more sensitive to tetracaine if, perhaps due to structural factors, L-type channels raise [Ca\textsuperscript{2+}], around coupled RyRs more than the Ca\textsuperscript{2+} released by coupled RyRs does at uncoupled ones.

The fact that propagation is limited in extent may be due to two factors: (1) if the cell is not overloaded with Ca\textsuperscript{2+}, the wave gradually dies out\textsuperscript{22}; and (2) when the wave reaches a region that released on the previous pulse, release may be less favored (see next section) and the wave will stop.

Miniwave Initiation Alternates From Pulse to Pulse

The probability that a given region shows the second wave-like phase of Ca\textsuperscript{2+} release is not random but tends to alternate from pulse to pulse. One explanation of alternans is that a large systolic Ca\textsuperscript{2+} release renders the SR less able to release on the next stimulus. This could arise via the following mechanisms: (1) the initial Ca\textsuperscript{2+} release may inactivate the Ca\textsuperscript{2+} release mechanisms,\textsuperscript{23} such that on the second beat, less is released. Inactivation will be less on the third beat, therefore giving a large release. (2) It may take time for the Ca\textsuperscript{2+} released on the first stimulus to be available again for release.\textsuperscript{24,25} (3) A large Ca\textsuperscript{2+} transient will result in a large Ca\textsuperscript{2+} efflux from the cell, thereby depleting the SR such that less Ca\textsuperscript{2+} is available for release on the next pulse. Evidence against this possibility comes from previous work on atrial myocytes where qualitative measurements of SR Ca\textsuperscript{2+} content during alternans show no change of SR Ca\textsuperscript{2+} content.\textsuperscript{26} In the present work, we have calculated the local changes of SR Ca\textsuperscript{2+} content (Figure 7A) and concluded that they are too small to account for the observed alternans. It should, however, be noted that the overall appearance of the alternans reported here depends on propagation of Ca\textsuperscript{2+} release. Previous work has shown that if cellular Ca\textsuperscript{2+} loading is below a certain threshold level then propagation does not occur.\textsuperscript{22,27} Therefore, small changes in content might determine whether the secondary release responsible for the appearance of alternans occurs.

Relationship Between Subcellular and Cellular Alternans

Alternation in the amplitude of the Ca\textsuperscript{2+} transient or contraction has been observed previously at both the whole-cell level or multicellular tissues.\textsuperscript{17,24,28,29} Recently, regions comprising several cells have been found to alternate in phase but out of phase with other regions.\textsuperscript{30} The present study shows that tetracaine or acidification can result in a small degree of alternans at the whole cell level (Figures 2 and 6). One novel result is that, under these conditions, alternating Ca\textsuperscript{2+} release is more obvious at the subcellular level where regions of the order of tens of microns alternate out of phase with each other. If this is to contribute to alternans at the tissue level, then Ca\textsuperscript{2+} release must be coordinated within and between cells. Even with a relatively small number of cells all showing subcellular alternations, the average signal would be quite uniform if the subcellular alternans were occurring randomly. It is not clear what that coordinating influence might be. It should not be forgotten, however, that at the level of the whole heart, factors such as alternation of end diastolic volume in addition to those of myocardial contractility may also be important.\textsuperscript{31}

Recent work comparing Ca\textsuperscript{2+} release in ventricular and atrial myocytes has reported alternans in response to inhibitors of glycolysis.\textsuperscript{26} Ca\textsuperscript{2+} release in ventricular cells was uniform, even when alternans were taking place. In contrast, we find a strong tendency of ventricular cells to show regional variations in Ca\textsuperscript{2+} release during alternans. The apparent uniformity seen previously may reflect the fact that line scans were made across the short axis of the cell, a distance that is small compared with the size of the regions we report.

Pathophysiological Implications

Depression of RyR opening produces marked local variability in the onset of the systolic Ca\textsuperscript{2+} transient. The observations
are strikingly similar to those seen in cells from infarcted hearts (cf., Figure 7 of Litwin et al30) and are consistent with the possibility that in those hearts there will be either a decrease in the number of RyRs32 or impairment in the coupling between the L-type channel and RyR,3 leading to an increase in the number of orphan RyRs. We also find that depression of RyR opening prolongs the duration of both the systolic Ca2+ transient and the resulting contraction. These effects arise from the variability in the time of Ca2+ release in different regions of the cell. A slowed decay of the Ca2+ transient is a common observation in heart failure in both humans and animal models33,34 and is generally attributed to reduced activity of SERCA. Although there are other ways in which the RyR can be involved in heart failure, including decreased SR Ca2+ content due to leaky, hyperphosphorylated RyRs,35,36 the present results at least raise the possibility that continuing Ca2+ release may also contribute to slowed relaxation.

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