UltraRapid Communication

Pacing-induced Heterogeneities in Intracellular Ca\(^{2+}\) Signaling, Cardiac Alternans, and Ventricular Arrhythmias in Intact Rat Heart


Abstract—Optical mapping studies have suggested that intracellular Ca\(^{2+}\) and T-wave alternans are linked through underlying alternations in Ca\(^{2+}\) cycling-inducing oscillations in action potential duration through Ca\(^{2+}\)-sensitive conductances. However, these studies cannot measure single-cell behavior; therefore, the Ca\(^{2+}\) cycling heterogeneities within microscopic ventricular regions are unknown. The goal of this study was to measure cellular activity in intact myocardium during rapid pacing and arrhythmias. We used single-photon laser-scanning confocal microscopy to measure Ca\(^{2+}\) signaling in individual myocytes of intact rat myocardium during rapid pacing and during pacing-induced ventricular arrhythmias. At low rates, all myocytes demonstrate Ca\(^{2+}\) alternans that is synchronized but whose magnitude varies depending on recovery kinetics of Ca\(^{2+}\) cycling for each individual myocyte. As rate increases, some cells reverse alternans phase, giving a dysynchronous activation pattern, even in adjoining myocytes. Increased pacing rate also induces subcellular alternans where Ca\(^{2+}\) alternates out of phase with different regions within the same cell. These forms of heterogeneous Ca\(^{2+}\) signaling also occurred during pacing-induced ventricular tachycardia. Our results demonstrate highly nonuniform Ca\(^{2+}\) signaling among and within individual myocytes in intact heart during rapid pacing and arrhythmias. Thus, certain pathophysiological conditions that alter Ca\(^{2+}\) cycling kinetics, such as heart failure, might promote ventricular arrhythmias by exaggerating these cellular heterogeneities in Ca\(^{2+}\) signaling. (Circ Res. 2006;99:e65-e73.)

Key Words: calcium transients ■ calcium alternans ■ subcellular alternans ■ arrhythmias

One of the most important clues to the mechanisms responsible for repolarization alternans was derived from the fact that action potential duration (APD) alternans occurs at the cellular level in intact heart.\(^1-3\) It is now widely accepted that T-wave alternans (TWA) on the surface ECG reflects tissue repolarization alternans at the level of the whole heart. In contrast to a purely electrophysiological explanation involving ion channel kinetics,\(^4,5\) evidence suggests that APD and T-wave alternans are in fact associated with changes in intracellular Ca\(^{2+}\) dynamics.\(^2,5-7\) The link between alternations in intracellular Ca\(^{2+}\) dynamics and TWA has recently been summarized\(^2\) as possibly arising from underlying alternans in Ca\(^{2+}\) cycling. Intracellular Ca\(^{2+}\) release enters into an alternating pattern based on the balance between the dynamics of Ca\(^{2+}\) release, reuptake, and recovery rates that induce oscillations in APD as a result of Ca\(^{2+}\)-sensitive conductances. Theoretically, a large contraction occurs as the result of a large release of Ca\(^{2+}\) from stores in the sarcoplasmic reticulum (SR), which would in turn cause a large inward Na/Ca exchange current (I\(_{\text{NCX}}\)) and a long APD. Because the large SR Ca\(^{2+}\) release would have the effect of temporary depletion of SR Ca\(^{2+}\) content, the next beat would activate a small Ca\(^{2+}\) release with a resulting small contraction and a small inward I\(_{\text{NCX}}\) contributing little to APD, which would then be short. Other Ca\(^{2+}\)-sensitive currents may also be activated that could either prolong or abbreviate APD, depending on transmembrane potential and whether or not they are present in a given species and tissue type.\(^6\)

The spatial organization of repolarization alternans has primarily been studied using optical mapping of voltage- and Ca\(^{2+}\)-sensitive dyes.\(^3,8-10\) Despite its advantages in allowing simultaneous study of electrical activation across the entire left ventricle (LV), the low signal-to-noise ratio of these dyes requires that each detector element records from hundreds to thousands of cells. The result is that cardiac activity cannot be measured at the cellular level, and consequently the heterogeneities that might exist between cells will be missed. If there are in fact disparities in Ca\(^{2+}\) signaling in neighboring

Original received May 5, 2006; resubmission received July 17, 2006; revised resubmission received August 15, 2006; accepted August 24, 2006.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/01.RES.0000244087.36230.bf
Ca\(_{\text{2+}}\) an inherent property of the normal cell-to-cell variation in heterogeneities within microscopic regions of ventricle are not yet known. Their existence could provide further evidence that is altered by increased rate. The goal of this study was to measure characteristics of Ca\(_{\text{2+}}\) transients; bright corresponds to systole, dark corresponds to diastole. C, X-t line-scan image acquired with the line placed across the 14 cells as indicated by the white arrow on B. Each myocyte in B is indicated in C with a green arrow on B. Each myocyte in B is indicated in C with a green arrow on B. Each myocyte in B is indicated in C with a green arrow on B. Each myocyte in B is indicated in C with a green horizontal line. The blue horizontal line indicates a particularly bright region of a capillary, which provides a landmark for relating line-scan to frame-scan images. The 2 Ca\(_{\text{2+}}\) transients recorded in C show simultaneous activation of all 14 myocytes in the image (labeled cells 1 to 14) and a typical intensity profile for average fluorescence (above).

cardiac myocytes, this type of behavior will be of considerable importance because it is likely to contribute to electrical nonuniformities in small ventricular regions or even single cells, thus setting the stage for arrhythmias. However, the patterns of cellular Ca\(_{\text{2+}}\) release in LV have not been investigated; therefore, the development of Ca\(_{\text{2+}}\) cycling heterogeneities within microscopic regions of ventricle are not yet known. Their existence could provide further evidence that the substrate for arrhythmias might be present as an inherent property of the normal cell-to-cell variation in Ca\(_{\text{2+}}\) dynamics that is altered by increased rate. The goal of this study was to measure characteristics of Ca\(_{\text{2+}}\) signaling in individual myocytes in intact ventricle that could contribute to the development of pacing-induced alternans and resulting arrhythmogenesis.

### Materials and Methods

The methods used in this study involve modifications of Langendorff perfusion of intact rat heart, first loaded with the Ca\(_{\text{2+}}\)-sensitive fluorescent dye fluo-4/acetoxyethyl ester (fluo-4AM), then paralyzed with cytochalasin D. Ca\(_{\text{2+}}\) transients were subsequently measured in individual myocytes on the left ventricular subepicardium using single-photon laser-scanning confocal microscopy. Because the details of our approach have not been described previously, we have included an extensive section in the online data supplement describing how these experiments were performed and analyzed.

### Results

#### Development of Synchronous Alternans

Figure 1A shows the region of the LV from which recordings were typically made, a 2D fluorescence intensity image of the left ventricular surface (Figure 1B) and the corresponding line-scan image (Figure 1C) from which high-resolution recordings of intracellular Ca\(_{\text{2+}}\) concentration were made. The square in Figure 1A indicates a typical recording site on the LV. Figure 1B shows an X-Y confocal frame-scan image of the cardiac surface with myocytes oriented in long bundles. The regions of bright fluorescence indicate the time and position of SR Ca\(_{\text{2+}}\) release transients during stimulation during the nearly 3 seconds required to construct this image.

To measure Ca\(_{\text{2+}}\) transients simultaneously in a number of myocytes with high temporal and spatial resolution, the scan line was placed across 14 cardiac myocytes on the left ventricular epicardial surface (white arrow in Figure 1B). The X-t line-scan image to the right of the frame-scan (Figure 1C) shows how cytoplasmic Ca\(_{\text{2+}}\) transients are measured in individual myocytes. The location of each myocyte is indicated by the green horizontal line (Figure 1B) during repetitive scanning along the line shown in Figure 1C. The average fluorescence for each scan from all cells is indicated in the intensity profile above and shows typical fluorescence intensity data, with the brightest intensity occurring at peak systole, followed by a fall in intensity during Ca\(_{\text{2+}}\) removal for the cytoplasm.

The normal behavior of cardiac myocytes at basal pacing rates (at BCL [basic cycle length]=500 ms) is shown in Figure 2A. This line-scan image shows 2 responses at the basal rate followed by a 10-second test train at BCL=260 ms, followed by the first response back at basal pacing. The last basal beat is followed by smaller transients in all myocytes and the ensuing test train demonstrates nominally uniform Ca\(_{\text{2+}}\) alternans in a large–small–large–small sequence until rapid pacing ends. Nearly all cells show some degree of alternans; however, there is a great degree of heterogeneity in the responses of individual myocytes. Figure 2B shows an expanded view of the last 4 rapid pacing beats and the first after return to basal rate. The variability in steady-state alternans magnitude from cell to cell is apparent in the profiles of 4 cells selected, ranging from nearly complete alternans (cells 2 and 12) to none at all (cell 5). All cells in alternans are in the same phase, demonstrating behavior termed "synchronous alternans." This form of alternans occurs over a range of BCLs such that the alternans ratio (AR=1−S/L, based on Ca\(_{\text{2+}}\) transient peak amplitude; where S indicates short and small transient and L, large and long transient) increases with decreasing BCL for each cell (Figure 2C). However, the BCL at which each cell develops alternans is variable, suggesting that the susceptibility to alternans onset differs between myocytes.

The relationship between BCL and AR for each cell was fitted to a sigmoid and the threshold cycle length (effective cycle length at AR=0.20 [ECL\(_{0.20}\)]) and the midpoint (ECL\(_{50}\)) were calculated as demonstrated in Figure 3A. To determine which characteristics of Ca\(_{\text{2+}}\) transients might predict sensitivity of each cell to alternans development, the values for ECL\(_{0.20}\) and ECL\(_{50}\) were plotted as a function of a number of properties of the Ca\(_{\text{2+}}\) transients at the basal rate of 500 ms (Figure 3B through 3E). The only reliable predictors of vulnerability to alternans were related to transient duration (Figure 3B) and Ca\(_{\text{2+}}\) reuptake rate, as indicated by the transient decay time (Figure 3C). In contrast, there was no
apparent relationship between vulnerability to alternans onset and rise time (Figure 3D), total Ca$^{2+}$ released (Figure 3E), magnitude of release, or time-to-peak (data not shown).

Development of Dyssynchronous and Subcellular Alternans

A different pattern of Ca$^{2+}$ signaling among neighboring myocytes occurs at higher pacing rates. Immediately after acceleration of BCL from 500 to 230 ms (Figure 4A), a number of cells developed alternans that is out of phase with the majority of cells in the field. The left expanded segment below the complete image in Figure 4A (white bar, Figure 4B) shows that the initial responses exhibit disorganized behavior, particularly for cells 3 and 4 as indicated by their respective intensity profiles. The right image (Figure 4C) is a temporal expansion of the last 4 rapid pacing beats and the first after return to the basal rate (teal bar in Figure 4A). Several myocytes are out of phase with others in the immediate vicinity, including cells 2, 5, 8, 10, and 12, as indicated by their respective intensity profiles. This “dyssynchronous

Figure 2. Cell-to-cell heterogeneity in the development of synchronous whole cell Ca$^{2+}$ alternans in epicardial myocytes in situ. A (top), Line scan image from Figure 1 corresponding to overall mean fluorescence intensity profile showing 2 Ca$^{2+}$ transients at the basal BCL=500 ms, followed by a rapid train at BCL=260 ms (10 seconds) and finally 1 transient on return to BCL=500 ms. B, Temporal and spatial expansion of image A (horizontal teal bar) showing the last 4 beats in the fast pacing epoch and one at basal rate. Mean fluorescence intensity profiles for cells 2, 5, 8, and 12 are shown at right, C, Graph of AR vs pacing BCL for each cell in this site. Data points for each cell are indicated by their assigned numeration and thin lines are their respective fitted sigmoid curves. The bold points, curve, and error bars are the mean±SEM for the group of cells in this site.

Figure 3. Predictors of cellular vulnerability to Ca$^{2+}$ alternans development. A, Graph of AR vs BCL for a cell fitted to a sigmoid curve showing calculation of ECL$_{20}$ (effective cycle length threshold for alternans onset) and ECL$_{50}$ (at half maximal alternans development). B, Graphs of ECL$_{20}$ and ECL$_{50}$ vs transient duration at 50% recovery (TD$_{50}$). N=172 myocytes from 11 hearts. C through E, Graphs of ECL$_{20}$ vs decay time, rise time, and total Ca$^{2+}$ release of the basal Ca$^{2+}$ transient, respectively.
alternans” may remain stable for the duration of the test train or may last only for several beats and become synchronous with surrounding myocytes. However, note that myocytes both distant from one another and immediately adjoining may demonstrate dyssynchrony.

Dyssynchrony among myocytes on the LV occurred with an overall incidence of 21% (119/575 myocytes in 31 sites from 18 hearts). These results demonstrate the very high degree of microscopic heterogeneity in Ca\(^{2+}\) cycling that occurs in a rate-dependent manner.

The recording in Figure 4B is a temporal and spatial expansion of cells 3 and 4 in the left lower image of Figure 4A (teal bar) and demonstrates a third distinct form of Ca\(^{2+}\) signaling that is evident in several cells in this image. Under these conditions, Ca\(^{2+}\) release also varies in different regions within both cells 3 and 4 during several cycles of rapid pacing. For example, during the forth cycle of the image shown in Figure 4D, the top parts of cells 3 and 4 show little Ca\(^{2+}\) release, whereas the bottom part shows prominent release. The opposite pattern of release then occurs in the fifth cycle. However, the spatial and temporal pattern of heterogeneous intracellular Ca\(^{2+}\) release changes throughout rapid pacing (Figure 4A). This intracellular alternans pattern illustrates a phenomenon known as “subcellular alternans.”\(^{11–13}\)

This figure demonstrates that the same conditions that promote dyssynchronous Ca\(^{2+}\) release between different myocytes simultaneously promotes heterogeneities in Ca\(^{2+}\) release within individual myocytes.

Subcellular Alternans in Intact Epicardium

To investigate subcellular alternans within individual myocytes of intact LV, we recorded line-scan images along the longitudinal axis of individual myocytes in the left ventricular epicardium (Figure 5). This approach allowed high spatial and temporal resolution Ca\(^{2+}\) imaging along myocyte length during stimulation. In addition, these hearts were stained with di-8-ANEPPS to outline the cell membranes, so that cell ends could be located during positioning of the line and during analysis. Figure 5A shows a 2D image of a single cell outlined by di-8-ANEPPS staining. The fluorescence intensity was high in this image because systole occurred during recording. The white box shows the cell outline, with the brightest portions appearing as lines along the cell ends (yellow). The scan line was placed from end to end (blue line, Figure 5B) as indicated by the dashed arrows showing the recording site. The top line-scan image in Figure 5C illustrates at basal BCL=500 ms, followed by a train of transients at a fast pacing BCL=220 ms, then a return to basal rate. D, Pacing at BCL=190 ms. E, Pacing at BCL=180 ms.
subcellular Ca\(^{2+}\) alternans at 35°C, indicating a similar incidence rate that was close to that found at room temperature. Overall, 9/14 (~64%) myocytes demonstrated subcellular alternans at 35°C, indicating a similar incidence rate to that observed during recording at the lower temperature.

Overall, there is a shift in their rate dependence of all 3 forms of alternans to shorter cycle lengths because Ca\(^{2+}\) cycling is highly sensitive to temperature, reflecting the shorter transient durations at 35°C. One result is an increase in rate sensitivity of synchronous alternans development with increasing rate at 35°C, so that more cells developed alternans with smaller changes in rate than at room temperature. Thus, hypothermia may have important influences on the exact stimulation rates at which the different alternans subtypes

Figure 6. Alternans subtypes at physiological temperature (35°C). A, BCL was reduced from basal (350 ms) to 200 ms during the test train. Intensity profiles during transverse scanning at right show fluorescence changes during last 4 beats at BCL=500 ms and the first spontaneous beat following the train in 3 representative myocytes. B, Pacing at BCL=140 ms in same site as A. C, Longitudinal recording of a single myocyte in the LV shows basal pacing at BCL=350 ms and pacing at BCL=130 ms for 10 sec. D, Summary graph of ECL\(_{20}\) vs transient duration at 50% recovery (TD\(_{50}\)) from experiments performed at room temperature (same data as in Figure 3; open circles) and at 35°C (filled circles; n=90 myocytes from 7 sites in 3 hearts). Asterisks denote the first 12 (A and B) and 6 (C) stimuli of test train.
subcellular alternans measured in stable spontaneous VT. The top image illustrates the variability in the spatial and temporal patterns of subcellular alternans in 2 complete cells and part of a third cell, whereas the bottom image illustrates the behavior within a single cell. Of 12 hearts in which VT was either induced or occurred spontaneously, 11 hearts demonstrated dyssynchrony within the site from which recordings were made. Longitudinal single-cell recordings were made in 9 of these hearts, and all demonstrated subcellular alternans in at least 1 cell in the visual field during VT. Typical examples of VT in multicellular sites of both rat and guinea pig heart (supplemental Videos 3 and 4, respectively) demonstrate simultaneous dyssynchrony and subcellular alternans during spontaneous VT. These movies demonstrate the highly complex Ca\(^{2+}\) dynamics that occur during arrhythmogenesis both among and within myocytes in microscopic regions of LV. At the present time, there are insufficient data to prove that the development of VT is mechanistically related to alternans under these experimental conditions. However, the occurrence of both dyssynchronous and subcellular alternans in nearly every heart that developed VT raises the possibility that these forms of heterogeneity may contribute to pacing-induced VT in this experimental model.

**Discussion**

These results demonstrate that the degree of heterogeneity in Ca\(^{2+}\) signaling at the cellular level is greater than expected from previous studies in which cellular behavior could only be inferred and not recorded directly.\(^3\)–\(^10\) Several previous reports have described the use of confocal microscopy in studying Ca\(^{2+}\) waves and transients in individual myocytes of the intact heart.\(^17\)–\(^22\) We found that at low rates, alternans developed that was uniform in phase but not in magnitude. As pacing rate increases, dyssynchronous Ca\(^{2+}\) release between cells and subcellular alternans within individual myocytes contribute to heterogeneity of cellular Ca\(^{2+}\) signaling, the extent of which is clearly seen during pacing-induced VT. Because Ca\(^{2+}\) release affects membrane conductances and therefore the cardiac action potential, it is possible that the dispersion of action potential duration and of refractoriness is much greater at the microscopic level than previously considered. However, electrotonic influences will serve to smooth the disparities that might accompany the high degree of Ca\(^{2+}\) cycling heterogeneities that occur between neighboring myocytes. Thus, we might speculate that Ca\(^{2+}\) alternans may be most arrhythmogenic when increasing APD dispersion at the macroscopic level (in the form of discordant alternans), whereas subcellular alternans may serve as a protective mechanism at still higher rates by diminishing the impact of coordinated regional Ca\(^{2+}\) overload producing triggered arrhythmias.

**Mechanism of Synchronous Alternans**

There has been a great deal of study recently about how oscillations in SR Ca\(^{2+}\) release can produce Ca\(^{2+}\) alternans in isolated myocytes.\(^23\)–\(^25\) One modeling study in particular provided strong theoretical support for a major role in alternans for the relationship between the diastolic SR Ca\(^{2+}\) load and its influence on Ca\(^{2+}\) release. A separate contribution
to alternans development and magnitude might also arise from the degree of Ca\(^{2+}\)-induced inactivation of the L-type Ca\(^{2+}\) current, which alternates with the level of cytoplasmic Ca\(^{2+}\) during Ca\(^{2+}\) alternans. However, these studies have largely ignored the role of Ca\(^{2+}\) transient duration in regulating the availability of Ca\(^{2+}\) for subsequent release with each cycle. Thus, a large and long (L) transient will be interrupted before SR Ca\(^{2+}\) content can recover at fast pacing rates, whereas small and short (S) transients will allow more complete recovery for the next cycle, which will then be large. One implication of this observation is that because Ca\(^{2+}\) transients in human heart failure\(^{26-27}\) and in animal models\(^{28-30}\) are prolonged, this effect could potentially increase the vulnerability to alternans onset.

Our results provide the first direct evidence that different cells in intact heart have different vulnerabilities to alternans development based on intrinsic properties of Ca\(^{2+}\) signaling within individual cells. These results are similar to recent observations in both dog\(^{31}\) and guinea pig\(^{32}\) and are consistent with the idea that the rate of Ca\(^{2+}\) cycling and, specifically, the duration of the Ca\(^{2+}\) transient might determine both the magnitude of and rate sensitivity to alternans. Moreover, this was the first demonstration of the variability in Ca\(^{2+}\) transients that exists at the microscopic level; previously, it has always been largely assumed that Ca\(^{2+}\) transients are uniform within each left ventricular region (eg, base versus apex) despite the fact that important differences exist between regions.\(^{1,3,9,10}\) Our observations demonstrate that this is not the case, and, furthermore, there is a high degree of heterogeneity in Ca\(^{2+}\) dynamics within microscopic regions of the LV at both low and physiological temperatures.

It is not yet clear how dysynchrony develops, but the result is that neighboring groups of cells and, in many cases, immediately adjoining cells are capable of developing alternans that is out of phase. The result is a much greater cell-to-cell variability in Ca\(^{2+}\) signaling at the microscopic level than was previously recognized. It is not yet known how or even if these microscopic heterogeneities in Ca\(^{2+}\) signaling might contribute to regionally discordant electrical alternans. However, dysynchrony could contribute to the substrate for reentrant arrhythmias, if its influence in the LV is sufficient in magnitude and distribution to contribute to a dispersion in refractoriness across the LV.

Subcellular Alternans, Cellular Ca\(^{2+}\) Heterogeneities, and Arrhythmias

It has only recently been shown that intracellular SR Ca\(^{2+}\) release can also go into oscillation in localized regions within individual myocytes.\(^{11,33}\) Part of the cell releases a large amount of Ca\(^{2+}\), whereas another cellular region released far less; the next activation produced the opposite pattern, causing a regional alternans within atrial cells but rarely in ventricular myocytes.\(^{12}\) A nearly identical phenomenon occurred during partial inhibition of ryanodine receptors (RyRs) in rat ventricular myocytes using low concentrations of tetracaine or acidosis.\(^{13}\)

The mechanism for subcellular alternans is most probably similar to that underlying Ca\(^{2+}\) alternans in general. One cellular region with slow Ca\(^{2+}\) reuptake is unable to respond to 2 successive beats during rapid pacing and so requires an additional cycle to replenish stores for large releases, whereas another region, the transient of which can recover more quickly, is able to follow each stimulus. We have also recorded instances in which different cellular regions simply oscillate completely out of phase with one another. The result is regional L/S alternations that are out of phase at different sites within the cell on a beat-to-beat basis.

When we studied the incidence of rate-dependent subcellular alternans development, we found that this is not an unusual phenomenon in the intact heart. In fact, nearly 2/3 of myocytes in intact rat LV demonstrated subcellular alternans at rates that produced pacing-induced VT and again during episodes of spontaneous VT. These observations raise, for the first time, the possibility that nonuniform intracellular Ca\(^{2+}\) release occurs during rapid pacing and arrhythmias.

Finally, the presence of both dysynchronous and subcellular alternans in nearly every heart that developed VT raises the possibility that alternans is involved in pacing-induced VT in this model. Conversely, if we had found that these forms of heterogeneity in Ca\(^{2+}\) signaling were absent during VT, it would be highly unlikely that they play any role in the arrhythmia. To our knowledge, these are the first direct measurements of Ca\(^{2+}\) signaling in single cells in intact heart during arrhythmias, and the results demonstrate an extraordinary heterogeneity in behavior at the level of individual myocytes during arrhythmias. Given that the electrical space constant far exceeds the behavior of individual myocytes, pacing-induced heterogeneities in ion channel activation and action potential in individual cells are unlikely to affect macroscopic electrical activity of the ventricular syncytium.

The smoothing effect of electrical current spread across many cells would, if anything, filter the most drastic cell-to-cell differences in Ca\(^{2+}\) signaling and resulting discrepancies in ion channel and action potential activation, thus minimizing their influence in arrhythmia development. However, a reduced space constant such as occurs in disease states, where intercellular communication is reduced,\(^{34,35}\) would minimize protection against nonuniformities in refactoriness, possibly promoting the development of re-entrant arrhythmias.

In the context of the relationship between transmembrane potential (V\(_m\)) and Ca\(^{2+}\) signaling heterogeneities at the cellular level, it is important to note that there are several reports of subcellular changes in V\(_m\) that might be relevant to the intracellular heterogeneities in Ca\(^{2+}\) signaling observed here. Several studies have found that cardiac cells do not depolarize uniformly during field stimulation, suggesting that a cellular “action potential” is subject to local changes in ion channel activation kinetics.\(^{36-38}\) The fact that we found Ca\(^{2+}\) signaling heterogeneities within individual myocytes of intact heart raises the possibility that different cellular regions might demonstrate local action potential variability depending on activation and recovery kinetics of Ca\(^{2+}\) sensitive conductances (I\(_{CaL}, I_{CaC} \) among others). A role for this type of nonuniform electrical activation has not been identified in arrhythmogenesis as yet. Furthermore, it is not known if the heterogeneities in cellular V\(_m\) are also present in intact heart because these studies were performed in isolated myocytes. However, it is known that current flow from cell to cell
depends on the uniformity of impedances during downstream conduction. Depolarizing ionic current as well as changes in resistance and capacitance between—and possibly within—myocytes can affect the spread and rise of depolarization. Thus it is possible that heterogeneities in cellular Ca\textsuperscript{2+} could also affect the rise time and shape of the action potential and the uniformity of propagation both between neighboring myocytes as well as within individual cells.

Lastly, it is worth mentioning that the observations of highly heterogeneous behavior in intact heart leading potentially to subcellular alternans have been described in theoretical studies published in recent years. This intracellular behavior is mathematically quite similar to that described for spatially discordant APD alternans in theoretical models across larger tissue regions. Alternatively, the behavior underlying subcellular alternans and possibly dyssynchronous alternans might best be described in terms of Turing-type instability within individual cells and between myocytes in small cardiac regions, respectively. It will be important in the future to analyze the cellular behavior observed here in terms of these theoretical considerations which could provide important insights into the basis for arrhythmia initiation and stabilization in the whole organ.

**Limitations of This Study**

One of the limitations of this study is that the arrhythmias were generated in hearts that were hypothermic. Low temperature affects ion channel behavior, intracellular Ca\textsuperscript{2+} cycling, and other factors that might contribute to arrhythmogenesis at nonphysiological temperatures. Ion channel activation is affected not only by a direct effect of cooling on kinetics but also reduced membrane fluidity, which alters channel environment in lipid membranes as well as membrane transport systems and therefore local transmembrane ionic balances. Conduction velocity is slowed because of reduced rapid Na\textsuperscript{+} current and gap junctional conductance. These factors will all contribute to arrhythmias at room temperature, making a definitive relationship between altered Ca\textsuperscript{2+} signaling and arrhythmias speculative at best.

One of the potential complicating factors in our interpretation of results is that cytochalasin D is required to prevent contraction. This agent is known to have direct effects on transients in addition to blocking contraction, raising the possibility that some of our observations about Ca\textsuperscript{2+} dynamics made in the presence of cytochalasin D might be influenced by its presence rather than occurring as a result of intrinsic properties of Ca\textsuperscript{2+} cycling in the heart. Cytochalasin D slows rise time and prolongs Ca\textsuperscript{2+} transient duration in rat ventricular myocytes but has little effect on dog heart (up to 80 µmol/L). Finally, the ability of cytochalasin D to slow Ca\textsuperscript{2+} transient kinetics was associated with a tendency to reduce the incidence and duration of reentrant ventricular arrhythmias in mouse heart. Overall, these data suggest that cytochalasin D is not affecting excitation/contraction coupling to a sufficient extent to explain the heterogeneities in Ca\textsuperscript{2+} signaling observed here.

**Sources of Funding**

A.H.K. was supported by the Fannie Penikoff Trust and is a scholar of the Feinberg Cardiovascular Research Institute.

**References**


**Disclosures**

None.


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_Circ Res._ 2006;99:E65-E73; originally published online September 7, 2006; doi: 10.1161/01.RES.0000244087.36230 bf

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Materials and Methods

Preparation of Whole-heart Perfusion. Rats were anesthetized with ketamine:xylazine (50 mg/kg : 5 mg/kg) or Na-pentobarbital (35-40mg/kg), i.p. The heart was rapidly removed, cannulated at the aorta and subjected to Langendorff (retrograde) perfusion at room temperature (~22°C). Perfusate solution composition was as follows (in mM): NaCl, 140, KCl, 5.4, CaCl2, 1.8, MgCl2, 0.5, NaH2P04, 0.4; NaHCO3, 22; glucose 10; equilibrated with blood-gas (95% O2 : 5% CO2); pH 7.35. A peristaltic pump was used to maintain the perfusate flow rate so that the diastolic pressure was kept at 50-70mm Hg throughout the experiments as monitored by an in-line high impedance pressure transducer (Bridge 8 bioamplifier, WPI). During an initial perfusion equilibration period of about 10 min, the heart was positioned in an experimental chamber on the stage of an inverted confocal microscope (Zeiss LSM 510 system, Jena, Germany) such that the LV is directed downward, facing the microscope objectives, and is between two Ag/AgCl2 wires used to monitor and record pseudo-ECGs.

The heart was immersed in and recirculated with a perfusate containing 15-20 µM of the non-ratiometric Ca2+ fluorescence dye, fluo-4AM (Molecular Probes) for 20-40 min and washed with normal solution for 10-15 min. In some experiments, membrane staining was accomplished using di-8-ANNEPPS (1-5 µM) during the last 5 min of dye loading. The heart was then recirculated with a perfusate containing the paralytic agent 50µM cytochalasin-D (and 50 µM latrunculin-B in some experiments) for the remainder of the experiment. Experiments were conducted at room temperature (23±1°C) or at 35°C as indicated. Platinum needle stimulating electrodes were inserted into the RV base and/or LV apex for delivery of rapid stimulation protocols. Hearts were paced at a basic cycle length (BCL) of 500 msec, unless otherwise stated.
Data acquisition. For fluorescence image acquisition, the heart was lightly pressed against the glass bottom of the chamber to provide a flat surface for image recording. Focal sites consisting typically of 15-20 myocytes (~500 µ²) at 2-3 cell layers below the LV epicardial surface were selected based on good electrical coupling and Ca²⁺ transient fluorescence signal strength. Fluo-4 excitation was achieved with a 25mW argon laser at 488 nm, and its fluorescence emission > 505 nm was collected using a lowpass filter. Line-scan recordings of confocal fluorescence images were acquired using the integrated Zeiss LSM Confocal Microscopy Software (V2.5) and either a 25x (NA 0.82) or 40x (NA 1.24) water objectives at a typical resolution of 512 pixels/line and a scan rate of 1.92 msec/line-scan. Photobleaching and phototoxicity by laser light was minimized by scanning at < 10% output transmission for ≤ 16 sec per image.

Pseudo-ECG signals were digitized at 1 kHz on-line with a 1384 Digidata A-D converter, and stored to a PC using PClamp8 software (Axon Instruments/Molecular Devices, Burlingame, CA). Synchronous confocal image and ECG acquisition was achieved by triggering (via TTL pulse) of ECG recording at the time of initiation of fluorescence image recording.

Rapid pacing protocol. Cellular Ca²⁺ alternans was induced by rapid pacing during abrupt acceleration from a basal BCL (500 msec) to a shorter test BCL for 10 sec, and then returned to basal BCL or intrinsic rate (no stimulation). This protocol was repeated until alternans was observed, at which point the test BCL was decreased by 10 msec until there was no alternating transient and/or arrhythmias occurred.

Data analysis. Figures show absolute fluorescence whereas statistical analyses were performed after fluorescence normalization to resting levels (F/F₀). No attempt was made to calibrate the fluo-4 fluorescence (F) to absolute [Ca²⁺], or to correct for autofluorescence. Using the Zeiss LSM software, each individual myocyte in an X-t
line-scan image field was visually identified in order to distinguish a series of cells joined laterally in a given recording site, aided by an X-Y frame-scan counterpart of that site. As shown in Figure 1, the long myocyte bundles of the LV subepicardium were separated laterally from the neighboring cells either by a brightly stained capillary or by dark patches of unstained tissue. The figure shows how the cell boundaries can be transposed to the line-scan image, often with the help of landmarks such as brightly loaded capillaries whose fluorescence intensity does not vary appreciably during pacing. This approach made it quite easy to distinguish between myocytes during transverse (multicellular) recording. Staining with di-8-ANEPPS was not used routinely in experiments with transverse recordings of multiple cells because of the significant spectral overlap with fluo-4, which interfered with quantification of basal transient characteristics and of changes during rapid pacing. Occasionally, it was difficult to determine if a particularly wide bundle (>20 µm) was in fact one or two cells. In this case, rapid pacing invariably revealed either uniform Ca^{2+} signaling behavior as characteristic of a single myocyte or distinct behavior indicative of multiple cells (highly non-uniform transients or complete block in one but not the other cell). In these rare instances (about 1 cell per 3-4 hearts) where an apparently single myocyte clearly demonstrates the behavior of two closely adjoined cells, the analysis was repeated for each member of that cell pair. In contrast, all longitudinal recordings of individual myocytes or cell pairs during investigation of pacing-induced changes in Ca^{2+} signaling within each myocyte were performed in di-8-ANEPPS stained hearts. This approach allowed easy identification of intercalated discs between myocytes within a cell bundle in order to be certain that each recording was made within the cytoplasm of 1-2 clearly identifiable cells.

Each myocyte was analyzed at all test cycle lengths. The corresponding Ca^{2+} transient fluorescence data were extracted and analyzed separately. Characteristics of
Ca\textsuperscript{2+} transients at a BCL of 500msec—peak amplitude, amplitude-time integral, rise-time (10-90% peak amplitude), decay-time (90-10% peak amplitude), rate of rise (+dF/dt) and decay (-dF/dt), transient duration (50% and 90% recovery or TD\textsubscript{50} and TD\textsubscript{90})—were determined using Matlab software to assess predictors of cellular Ca\textsuperscript{2+} alternans susceptibility. Alternans ratios (AR) were calculated as $1 - \left( \frac{\text{Small}}{\text{Large}} \right)$ transient magnitude\textsuperscript{16} at steady-state (end of 10-sec test train). Because Ca\textsuperscript{2+} transients were measured at the level of individual myocytes, the signal was small compared to that of voltage-sensitive dye mapping studies. Consequently, an AR of 0.2 was considered to be the threshold for cellular Ca\textsuperscript{2+} alternans. Sigmoid curves were fitted to plots of alternans ratio vs. test BCL in order to calculate the Effective Cycle Lengths at which 20% (ECL\textsubscript{20}) and 50% (ECL\textsubscript{50}) alternans were achieved. This approach allowed the calculation of the cycle lengths at which the threshold and half-maximal alternans values are achieved, respectively, as measures of susceptibility or vulnerability of individual myocytes to the development of alternans. Pseudo-ECG recordings were filtered off-line at 15-100Hz, examined and graphically prepared via pClamp8 software.

Elimination of motion artifacts. One of the critical limitations of this approach is that the heart must be immobilized in order to achieve accurate recordings of Ca\textsuperscript{2+} signaling at the cellular level. This was accomplished using cytochalasin D which interferes with the polymerization of actin, thus preventing contraction. Since the tolerance for motion using this approach is so small (<2\textmu m), it is essential to abolish contraction completely. This can be done using cytochalasin D but not BDM which, at concentrations necessary to block contraction (~50mM), had profound effects in pilot studies to slow conduction, prolong the QRS complex and reduce Ca\textsuperscript{2+} transient amplitude. None of these changes occurred with cytochalasin D in intact heart, making it a better agent for this purpose. Even small amounts of residual contraction were sufficient to preclude reliable
measurement of transients in individual myocytes. Figure S1 shows a site with approximately 12 myocytes that is moving about 10µ with each stimulus (BCL=240msec). Since motion changes not only the X-Y position of each cell but also the focal plane in the Z dimension, it is not possible to be certain that changes in fluorescence reflect changes in intracellular Ca\(^{2+}\) concentration and not simply movement of the cell out of focus. Consequently, images such as these were excluded from analysis. This image was recorded from a heart exposed to 40µM cytochalasin D; movement was eliminated in nearly all cases during exposure to 50µM. If any motion remained, an additional 10µM was added to the superfusate, which was always sufficient to stop motion completely. However, it is important to note that some of the movies included in the Supplementary Material were recorded in hearts that were not completely paralyzed. These recordings are included to demonstrate the heterogeneities in cellular Ca\(^{2+}\) signaling during rapid pacing in a more dramatic fashion than can be seen in line-scan images. However, none of the images from these movies was analyzed for single cell behavior, in part because sampling rate is too low and in part because of these motion artifact issues.

**Statistics.** All data are presented as mean ± SEM. Comparisons of sample means are performed using paired and unpaired Student’s t-test. If multiple comparisons are necessary, statistical significance is determined using an analysis of variance of repeated measures whenever possible with Bonferroni’s correction for multiple comparisons and Student-Newman-Keuls secondary tests. If data are unpaired, then a simple ANOVA is performed using the same post-hoc analyses. Statistical significance was assigned at \(p < 0.05\).
References


Legends for Supplementary Material

**Figure S1.** Typical Recording of Ca2+ Transients in Multiple Epicardial Cells When Cardiac Motion is Not Completely Abolished by Cytochalasin D. This image was acquired during stimulation at BCL=240msec in the presence of 40mM Cytochalasin D, which was insufficient to block all contraction. Images such as these were not analyzed because of changes in focal plane during stimulation.

**Movie #1. Movie of Subcellular Alternans.**

The movie begins with pacing at a BCL=500msec then abruptly decreases BCL to 240msec for 10sec followed by a return to the basal pacing rate. Stimulation at BCL=500msec demonstrates uniform spread of activation across all cells in the visual field. Within several cycles of increased pacing rate, stable subcellular alternans develop that are maintained throughout the remainder of the test train. Immediately after the return to 500msec, there are several cycles in which a single myocyte demonstrates a circular wave radiating from a point source inside the cell (upper left quadrant). All cells then show normal activation during maintained pacing at the basal rate. Frame rate was 9/sec for all movies. Scale of the image is 331µ per side.
Movie #2. Movie of Combined Dyssynchronous and Subcellular Alternans.

The move begins with pacing at a BCL=500msec then decreases to BCL=200 msec for 10 sec followed by a return to BCL= 500msec. During rapid pacing, many cells are completely blocked (AR=1); however several cells around the periphery give full responses that are out-of-phase with most of the cells in the image (dyssynchronous alternans). In the middle of the image, numerous myocytes show small regions of Ca2+ release within the cell on alternate cycles (subcellular alternans). All forms of alternans disappear immediately upon return to the basal pacing rate. Image scale is 353µ per side.

Movie #3. Movie of Combined Dyssynchronous and Subcellular Alternans During Spontaneous VT in Rat Heart.

A stable episode of VT was maintained for 2 minutes following rapid pacing. The VT was characterized by highly complex Ca^{2+} signaling, including simultaneous dyssynchronous and subcellular alternans. Image scale is 314µ per side.

Movie #4. Movie of Complex Ca^{2+} Dynamics During Spontaneous VT in Guinea Pig Heart.

A stable episode of VT was maintained for 3 minutes following rapid pacing. Simultaneous dyssynchronous and subcellular alternans were present throughout the episode of VT. Image scale is 184µ per side.
Example of Heart Movement
When [Cyto D] is Too Low