Multiple Defects in Intracellular Calcium Cycling in Whole Failing Rat Heart

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Number of words in abstract: 250
Total number of words: 6261
Number of figures: 8
Number of references: 35
Number of tables: 0

Abstract

Background: A number of defects in excitation-contraction coupling have been identified in failing mammalian hearts. The goal of this study was to measure the
defects in intracellular Ca\textsuperscript{2+} cycling in left ventricular epicardial myocytes of the whole heart in an animal model of congestive heart failure.

**Methods and Results:** Intracellular Ca\textsuperscript{2+} transients were measured using confocal microscopy in whole rat hearts from age-matched Wistar-Kyoto (WKY) control rats and Spontaneously Hypertensive Rats (SHR) at \sim 23 months of age. Basal Ca\textsuperscript{2+} transients in myocytes in SHRs were smaller in amplitude and longer in duration than WKYs. There was also greater variability in transient characteristics associated with duration between myocytes of CHF than WKY controls. Approximately 21% of CHF myocytes demonstrated spontaneous Ca\textsuperscript{2+} waves compared to very little of this activity in WKY. A separate population of SHR myocytes showed Ca\textsuperscript{2+} waves that were triggered during pacing and were absent at rest (triggered waves). Rapid pacing protocols caused Ca\textsuperscript{2+} alternans to develop at slower heart rates in CHF.

**Conclusions:** Epicardial cells demonstrate both serious defects and greater cell-to-cell variability in Ca\textsuperscript{2+} cycling in CHF. The defects in Ca\textsuperscript{2+} cycling include both spontaneous and triggered waves of Ca\textsuperscript{2+} release, which promote triggered activity. The slowing of Ca\textsuperscript{2+} repriming in the sarcoplasmic reticulum is probably responsible for the increased vulnerability to Ca\textsuperscript{2+} alternans in CHF. Our results suggest that defective Ca\textsuperscript{2+} cycling could contribute both to reduced cardiac output in CHF and to the establishment of repolarization gradients, thus creating the substrate for reentrant arrhythmias.

Key words: Ca\textsuperscript{2+} transients – Ca\textsuperscript{2+} waves – confocal microscopy –Ca\textsuperscript{2+} alternans – ventricular tachycardia
Introduction

Several defects in cardiac excitation-contraction (E-C) coupling have been identified in myocytes from failing hearts that could contribute to the mechanical and electrophysiological dysfunction causing pump failure and fatal arrhythmias. Studies in animal models and human hearts have shown that congestive heart failure (CHF) is associated with a loss of t-tubules, leading to anatomical and functional dissociation between electrical activation of the sarcolemma and release of Ca\(^{2+}\) from sarcoplasmic reticulum (SR). Therefore, increasing number of Ca\(^{2+}\) release units (CRUs) become isolated from their normal trigger—the L-type Ca\(^{2+}\) channel—and are activated by Ca\(^{2+}\) diffusion from neighboring CRUs rather than by Ca\(^{2+}\) channels. The ensuing delay in activation of these “orphaned” ryanodine receptors (RyRs) may cause a poorly coordinated release of Ca\(^{2+}\) along the cell length, leaving a highly fractionated cellular wave front during Ca\(^{2+}\) release. In combination with a slowing in Ca\(^{2+}\) reuptake due to reduced activity of the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), reduced rates of Ca\(^{2+}\) cycling are likely to contribute to diminished cellular contractile performance in CHF. Interestingly, increased action potential (AP) amplitude alternans, T-wave alternans and ventricular arrhythmias have been reported to be sensitive to altered Ca\(^{2+}\) cycling and are unrelated to action potential duration restitution.

Since all of these observations have been made in myocytes isolated from failing hearts, no information is available about how prevalent defective Ca\(^{2+}\) cycling is at the cellular level or what forms these defects may take in whole heart. Consequently, there is no basis upon which to extrapolate defects in function in isolated myocytes to changes in behavior in the whole heart.
The goal of this study was to investigate how cellular Ca\textsuperscript{2+} dynamics are altered in a CHF model. In the SHR model, the development of spontaneous hypertension occurs at 2-3 months of age and compensatory mechanisms, primarily hypertrophy, slow the development of overt CHF until after 20 months. We tested the hypothesis that defects in intracellular Ca\textsuperscript{2+} cycling in heart failure are responsible not only for reduced contractile function but also for the development of both triggered and reentrant arrhythmias. The experimental approach involves the use of confocal microscopy to measure intracellular Ca\textsuperscript{2+} dynamics in left ventricular (LV) epicardial myocytes of whole hearts\textsuperscript{8}. We used this approach to determine if defects in Ca\textsuperscript{2+} cycling are exhibited in myocytes of the intact epicardial surface (as opposed to isolated myocytes from various cardiac regions), what forms they take, if they are manifested as both triggered and spontaneous Ca\textsuperscript{2+} release and how uniform this defective behavior is in whole heart.
Methods

Briefly, hearts from male WKY and SHR rats (~21 months old) were excised and maintained on a Langendorff apparatus. The heart was placed in an experimental chamber on the stage of a confocal microscope and loaded with three successive additions of fluo-4AM (Invitrogen, 10-15μmol/l, 20 minutes each) in re-circulating Tyrodes solution. Dye was then washed for 10 minutes with normal perfusate. Solution was then re-circulated with Cytochalasin-D (50μmol/l) to block contraction for the remainder of the experiment and maintained at 25°C. Laser scanning of LV epicardial sites was accomplished with an LSM510 laser scanning confocal microscope (Zeiss Instruments) and allowed measurements of intracellular Ca2+ transients in individual myocytes during rapid pacing protocols. The scan line was usually placed across the short axis of 10-20 myocytes in a recording site on the epicardial surface using either a 25x (NA 0.82) or 40x (NA 1.2) water immersion objective. The scan line (512 pixels) was repeated at a sampling rate of 1.92msec/line. Data were collected during pacing protocols that consisted of constant basal pacing (basic cycle length BCL=700msec) of which the last 4 beats were recorded, followed by a 10-second train of rapid pacing at CLs ranging between 600-100msec followed by a 4 second pause before resumption of basal pacing. Data analysis was performed on each myocyte from each image during each test train. Characteristics of basal Ca transients were measured using MatLab and pCLAMP8 software. Alternans ratio was measured as 1 – (Small/Large) at the end of the 10-second test train. Restitution of SR Ca release was measured as the fractional recovery of the test beat compared to the last basal beat before the test interval. All data are presented as mean+SEM and data were compared using paired Students t-
tests. Differences between sample means were considered significant if p<0.05. Details of these methods are included in the Supplementary Material and have been published elsewhere\(^9\).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

*Characteristics of Ca\(^{2+}\) transients during basal pacing*

Typical recordings of multicellular sites are shown in *Figure 1A*, in which basal pacing evoked good electrical coupling and simultaneous activation of Ca\(^{2+}\) transients in all cells of a WKY heart. All myocytes show rapid Ca\(^{2+}\) release in response to stimulation and then decay of Ca\(^{2+}\) to resting levels as Ca\(^{2+}\) is removed from the cytoplasm. The average fluorescence for all cells is shown in the intensity profile plot above the image and indicates rapid rise and reuptake typical of normal heart. The profiles of two selected myocytes are shown to the right of the image and indicate normal Ca\(^{2+}\) transients typical of isolated myocytes and of normal whole heart. Also, small regions without fluorescence transients denote intercellular spaces that are either out of the focal plane or represent regions of connective tissue separating columnar cell bundles.

The recording from the SHR heart (*Figure 1B*) displays several important differences in cell function and anatomy compared to normal heart. First, peak release was less and both the rising and falling phases of the transients are slower, giving smaller and longer transients. Second, there is increased heterogeneity between myocytes, particularly in transient magnitude and duration. Third, there are more and wider non-fluorescent regions in SHR, indicating the presence of more fibrous tissue and collagen deposition. Finally, spontaneous Ca\(^{2+}\) waves are present in several cells (arrows) that either precede or follow the regular stimulus. These Ca\(^{2+}\) waves are not present in all cells but occur in certain myocytes either spontaneously or after a delay following normal activation, indicating an apparent randomness of activation rather than
the normal triggering expected from typical spontaneous Ca\textsuperscript{2+} release arising from Ca\textsuperscript{2+} overload. Overall, we found spontaneous Ca\textsuperscript{2+} waves in an average of 21.5±3.4% of myocytes in 15 sites with an overall incidence of 33/164 myocytes (20.1%, 3 SHR hearts) compared to 1/200 myocytes in 14 control sites (0.5%, 3 WKY hearts).

The average fluorescence profile (above) shows a slowing in both the rise and decay rates, typical of Ca\textsuperscript{2+} cycling defects in CHF. When transients from individual myocytes are examined, there is clearly a wide variability in behavior, reflecting some relatively normal transients, transients showing grossly delayed release and time to peak as well as spontaneous waves that affect both timing and magnitude of Ca\textsuperscript{2+} release between stimuli in ways that also contribute to the whole site Ca\textsuperscript{2+} cycling profile.

Analysis of the Ca\textsuperscript{2+} transient characteristics responding to basal stimulation is shown in Figure 2. There were decreases in peak magnitude and total amount of Ca\textsuperscript{2+} released, consistent with previous reports of reduced Ca\textsuperscript{2+} release in this model of CHF\textsuperscript{5}. Furthermore, there was a slowing of the maximal release rate (+dF/dt) and rise time as well as all characteristics relating to transient duration at 50% and 80% of recovery (TD\textsubscript{50} and TD\textsubscript{80}) and decay time, also consistent with previous findings in isolated myocytes.

We further investigated the characteristics of cellular Ca\textsuperscript{2+} transients by analyzing intercellular variability within a recording site in the form of heterogeneity indices (HI, measured as standard deviation within the recording site). These characteristics show significant differences in distribution within microscopic regions of the LV (Figure 3). All properties that determine the overall duration of the transient (rise and decay times as well as TD\textsubscript{50} and TD\textsubscript{80}) vary significantly more in SHR than in WKY hearts. Thus, not
only are there significant differences in the magnitude of these properties, but the cell-
to-cell variability is greater in SHR than WKY controls.

*Basis for cell-to-cell variability in Ca\textsuperscript{2+} transient characteristics*

It has been reported that myocytes from CHF hearts, including the SHR model, show defective E-C coupling in both the release and reuptake phases of the Ca\textsuperscript{2+} transient. Figure 4A shows that Ca\textsuperscript{2+} transients recorded during basal pacing along the long cell axis in two myocytes from a WKY heart rise rapidly to a sharp peak, then decline smoothly during Ca\textsuperscript{2+} reuptake, behavior that is fairly uniform for both myocytes. In contrast, the transients from the adjoining myocytes in the SHR heart (Figure 4B) show characteristics of defects in both release and reuptake. Regions of Cell 1 show delayed release, a prolonged time to peak and highly fragmented Ca\textsuperscript{2+} reuptake such that cellular regions showing slow release are also slow to recover while those with rapid release recover quickly. These characteristics are also seen in the fluorescence profile (above) showing slow rise, long time to peak and slow recovery of cytoplasmic Ca\textsuperscript{2+} levels during decline of the transient.

The adjacent Cell 2 (Figure 4B) shows relatively normal E-C coupling with uniform rapid release throughout the cell as well as a normal pattern of Ca\textsuperscript{2+} reuptake. Note, however, that despite its apparently normal appearance, Ca\textsuperscript{2+} release is in fact slower as is the time to peak compared to WKY (Figure 4A), giving a more rounded rising phase and peak as seen clearly in the fluorescence profile below.

These data address why there is increased heterogeneity in CHF rats compared to controls. Immediately adjoining myocytes show greater intercellular variability in Ca\textsuperscript{2+}
cycling in CHF than normals, thus accounting for the significantly greater HI for these parameters (Figure 3). The result is an overall slowing of Ca\textsuperscript{2+} release and prolongation of reuptake which reflects the average activity of a highly heterogeneous cell population with relatively “normal” transients immediately adjoining myocytes showing gross defects in Ca\textsuperscript{2+} handling.

**Defective Ca\textsuperscript{2+} handling during rapid pacing in CHF**

Since Ca\textsuperscript{2+} transients contribute to inward currents and action potential duration, it is important to know how local Ca\textsuperscript{2+} cycling is affected by increasing rate, especially under conditions known to be highly arrhythmogenic such as in CHF. The effects of increasing rate on Ca\textsuperscript{2+} transients in CHF are shown in Figure 5. The linescan image in Figure 5A shows transients activated at a site containing 10 myocytes interspersed with extensive fibrous tissue. All respond with fairly uniform transients to each stimulus delivered at basal pacing. Note that some – but not all - myocytes show signs of defective E-C coupling, with fractionated release and heterogeneous reuptake of Ca\textsuperscript{2+}. For instance, the selected cells shown at expanded scale in Figure 5B show fairly normal transients (Cell 9) in a cell adjoining a myocyte with clearly defective E-C coupling (Cell 10), even at BCL=700msec. However, the impairments in Ca\textsuperscript{2+} dynamics apparent in Cell 10 are enhanced at a higher heart rate (BCL=390msec, Figure 5C). The exaggerated fractionation of Ca\textsuperscript{2+} release causes a much greater delay in Ca\textsuperscript{2+} release in the cell center, which is consistent with a disruption of the t-tubule system and delay of voltage activation of orphaned RyRs in the cell core\textsuperscript{5}. The result is Ca\textsuperscript{2+} release similar to that observed in atrial myocytes\textsuperscript{9} showing the U-shaped activation
pattern characteristic of Ca\(^{2+}\) -induced Ca\(^{2+}\) release arising from Ca\(^{2+}\) diffusion from the sarcolemma inward (Figures 5B-C). Furthermore, the late release in the cell core during Beat 1 (Cell 10, Figure 5C) causes subcellular alternans as evidenced by a greatly diminished release of Ca\(^{2+}\) in Beat 2, then a continued alternation of Ca\(^{2+}\) release on subsequent beats. The result is an unstable alternans pattern arising from subcellular alternans, especially apparent in the duration of the transient in Cell 10 (profile below). Note that even Cell 9 shows some alternans in intensity and, to a lesser extent, in duration on alternating beats (profile above).

Ca\(^{2+}\) alternans development in CHF

One of the characteristics of many human CHF patients is an increased sensitivity to the development of T-wave alternans\(^{10}\), especially during exercise \(^{10-12}\). Because of the close association between Ca\(^{2+}\) and T-wave alternans, we investigated the possibility that SHR animals in CHF might also be more susceptible to Ca\(^{2+}\) alternans development during rapid pacing.

Figure 6A shows that decreasing BCL to 400msec caused little if any Ca\(^{2+}\) alternans in a WKY heart at steady-state at the end of rapid pacing. When BCL was reduced to 240msec, nearly all myocytes in the WKY heart showed alternans (Figure 6B). In contrast, a number of myocytes in the SHR heart showed alternans even at BCL=400msec (Figure 6C) whereas all myocytes showed alternans at BCL=340msec (Figure 6D). When the cycle length dependence of alternans ratio (AR = 1 / Small/Large\(^{13}\)) was plotted for all cells in one WKY site (Figure 6E), there was some variability between myocytes but the threshold for alternans was less than about 350
msec with a mean for the midpoint (estimated cycle length at 50% alternans, ECL₅₀) of 256±2.7 msec. The SHR site showed Ca²⁺ alternans at slower rates, with the threshold (20% alternans) for all cells beginning at about BCL=550msec in this site with an average midpoint for all myocytes of BCL=395±4.2 msec. The summary data show that the midpoint for rate-dependent development of alternans was significantly greater in CHF (341±6.0 in SHR compared to 271±5.8 msec, p<0.001, n= 116 myocytes in 8 sites in 3 hearts for SHR and 102 myocytes in 7 sites in 3 hearts for WKY), indicating a shift of 70msec in alternans vulnerability in CHF. The threshold for alternans development for all sites (ECL₂₀) also occurred at significantly slower rates in CHF (414±10.5 in SHR compared to BCL=340±13.2 msec in WKYs, p<0.001).

In addition to a greater rate-sensitivity to alternans development in CHF, there was also greater variability within each site in CHF hearts. The heterogeneity indices for both the midpoint (21±2.4 msec in WKY compared to 42±7.2 msec in SHR, p<0.02, n= 6 sites in 3 hearts in WKY and 8 sites in 3 hearts for SHR) and threshold (27±5.7 msec in WKY compared to 77±11.4 msec, p<0.001) of alternans development showed that there was greater intercellular variability for Ca²⁺ alternans development within each CHF site compared to normal hearts.

One of the determinants for the development of Ca²⁺ alternans is the rate of recovery of SR release. **Figure 7A** shows that individual myocytes in WKY rats demonstrated a rapid recovery of Ca²⁺ release with increasing interval, reaching nearly full recovery in most cases by 500msec. In contrast, the rate of restitution in the SHRs (**Figure 7B**) was so slow that it was not possible to test intervals short enough to allow myocytes to show the full time course of restitution without inducing VT. Consequently,
it was not possible to quantify the restitution in most myocytes from CHF hearts. However, it is clear that there is a dramatic slowing of restitution of SR Ca\(^{2+}\) release in CHF and that this slowing contributes to the development of Ca\(^{2+}\) alternans at slower heart rates.

Finally, the cycle-length dependency for VT induction was shifted to much longer cycle lengths in SHR hearts (BCL=176±14.6 in WKYs compared to 365±33.0 msec in SHRs, p<0.001, n= 4-5). In addition, the cycle-length threshold for intercellular discordant (or dyssynchronous\(^8\)) alternans was significantly longer in SHR (260±14.6 msec compared to 427±19.6 msec, p<0.001, n= 9-11 sites) with a similar increase in vulnerability to the development of subcellular alternans in SHR animals (287±7.5 compared to 468±17.7 msec, p<0.001, n=9-12 sites). These increases in rate sensitivity to both forms of complex Ca\(^{2+}\) cycling occurred with little change in overall incidence, with 9/14 (64%) of WKY sites compared to 11/15 (73%) of SHR sites demonstrating intercellular discordant alternans; while 10/14 sites (71%) of sites in WKY compared to 12/16 (75%) of sites in SHR developed subcellular alternans.

**Triggered Ca\(^{2+}\) waves**

In addition to spontaneous Ca\(^{2+}\) waves, an additional and distinct type of Ca\(^{2+}\) release event was observed in nearly all sites studied in CHF hearts. Some myocytes showed extremely weak Ca\(^{2+}\) release during normal activation but periodic Ca\(^{2+}\) waves in those cells showed that a large Ca\(^{2+}\) release was possible in these cells. More importantly, these waves did not occur spontaneously but were in fact triggered in response to normal electrical stimulation. The single SHR myocyte shown in Figure 8A
demonstrates weak Ca\textsuperscript{2+} transients in response to normal stimulation, but large Ca\textsuperscript{2+} waves occurred throughout the stimulation period (red arrows). This activity was abolished when stimulation was terminated and careful examination of the small transients evoked during rapid pacing shows that the central region of this cell was capable of small local transients from which Ca\textsuperscript{2+} waves originate and spread (“triggered Ca\textsuperscript{2+} waves”). This unique form of triggered Ca\textsuperscript{2+} release occurred in at least one myocyte in each of 11 of 20 sites (55%) in 3 SHR hearts. The image in Figure 8B shows transverse recordings of two neighboring myocytes from a failing heart in which Cell 1 demonstrates nearly normal E-C coupling while the adjoining Cell 2 shows triggered waves interspersed among very small and poorly coordinated Ca\textsuperscript{2+} transients. Supplementary Figure S1 shows that many myocytes in some sites demonstrate triggered Ca\textsuperscript{2+} waves during stimulation at basal pacing that are absent when pacing ceases. At the beginning of the movie, there are small Ca\textsuperscript{2+} release transients in all cells in the visual field even though many of the cells are also showing Ca\textsuperscript{2+} waves. When stimulation stops, all activity ceases for several seconds, followed by a few spontaneous waves in some cells immediately before pacing is re-started. In contrast, Figure S2 shows a movie from an age-matched WKY rat during basal pacing followed by a test train at BCL=360msec for 10 seconds then a pause. Ca\textsuperscript{2+} cycling is unremarkable during rapid pacing and shows no alternans or waves during stimulation. Several cells show spontaneous waves during the pause after rapid stimulation which is typical of normal rat myocardium following rapid pacing.

These data demonstrate a novel form of defective SR Ca\textsuperscript{2+} release that occurs in addition to that previously described in CHF. This form of Ca\textsuperscript{2+} release is poorly
coordinated between myocytes that are interspersed among normal neighbors, likely contributing to reducing contractile efficiency in whole heart. Moreover, the wave-like activity is likely to promote the triggered activity reported to develop in CHF.
Discussion

There have been numerous reports about defects in cellular Ca\(^{2+}\) handling in a variety of animal models of CHF and in human disease. Recent work has focused on the fractionation in the Ca\(^{2+}\) release wavefront that reduces the efficacy of the Ca\(^{2+}\) trigger for E-C coupling and occurs as the result of the loss of t-tubules in CHF\(^1,2,4,5\). The result is a population of “orphaned” RyRs in CHF, including that described in the SHR model, whose activation is delayed during the Ca\(^{2+}\) transient\(^5\). All of these studies were performed in isolated myocytes so it has not been possible to get a picture of how frequent these cellular changes are or how cell-to-cell interactions reflect dramatic changes in function.

Our results demonstrate that this defect in E-C coupling has a number of consequences in whole heart. First, there is a high incidence of myocytes showing abnormal Ca\(^{2+}\) handling, ranging from mild slowing of release and reuptake to severe abnormalities in Ca\(^{2+}\) cycling. Second, there is an enormous variability between myocytes so that cells showing severe defects reside in the midst of myocytes showing only mild changes in Ca\(^{2+}\) cycling. Third, spontaneous Ca\(^{2+}\) waves are common in CHF myocytes, raising the likelihood of triggered arrhythmias. Fourth, the rate dependence for Ca\(^{2+}\) alternans is shifted to much slower heart rates, probably because of a slowing in restitution of SR Ca\(^{2+}\) release and which could be responsible for increased T-wave alternans and reentrant arrhythmias found in the setting of CHF. Finally, Ca\(^{2+}\) cycling may become so defective that Ca\(^{2+}\) overload in the few remaining viable CRUs triggers a small Ca\(^{2+}\) release that then causes Ca\(^{2+}\)-induced Ca\(^{2+}\) release by diffusion along the cell length in the form of a triggered wave. Furthermore, these defects are distributed
unevenly across the ventricle and contribute to diminished pump function and enhanced arrhythmogenic substrate. We speculate that the result is less efficient Ca\(^{2+}\) cycling and cardiac contraction and an increased likelihood of both triggered and reentrant arrhythmias generated at slower heart rates in CHF than normals.

**Altered E-C coupling in CHF**

Many changes in E-C coupling have been reported in animal models and in patients with CHF. Ca\(^{2+}\) reuptake is slowed, possibly a result of reduced SERCA2a expression\(^6\). The Na-Ca exchanger (NCX) is upregulated which may compensate for reduced SERCA2a function\(^{14,15}\) and increased RyR2 phosphorylation has been reported, although the extent and sites of this phosphorylation are controversial\(^{16,17}\). SR Ca\(^{2+}\) leak increases, possibly as a consequence of RyR2 phosphorylation\(^{16,18}\). There may also be increased Na\(^+\) influx and intracellular Na\(^+\) accumulation\(^{19-21}\), increasing internal Ca\(^{2+}\) and SR Ca\(^{2+}\) load. Finally, many models of CHF demonstrate disorganization of the t-tubule system which could provide an anatomical basis for fragmentation of SR Ca\(^{2+}\) release \(^2-5\).

Previous studies in isolated cardiac myocytes from CHF in SHR animals have shown elevated RyR2 phosphorylation\(^{18}\), an overall slowing of Ca\(^{2+}\) reuptake and transient duration and loss of t-tubules which leads to orphaned RyRs and loss of viable CRUs\(^5\). We found that defective Ca\(^{2+}\) cycling is highly variable even among neighboring myocytes, with a severity sufficient in about 20% of myocytes to evoke spontaneous Ca\(^{2+}\) waves, even at slow heart rates. Furthermore, the problems in Ca\(^{2+}\) cycling are exaggerated at faster rates, which is a particular problem in CHF patients.
whose resting rates are typically higher than in normals. The result is reduced pump efficiency, increased T-wave alternans and increased likelihood of arrhythmias. Interestingly, there is also a recent report that AP amplitude alternans is associated with TWA and ventricular arrhythmias but not AP duration (APD) restitution, underscoring the link between Ca2+ cycling defects and arrhythmias that are independent of APD restitution. Finally, we found that defective Ca2+ release in the form of both triggered and spontaneous waves is more prevalent in CHF. It is known that Ca2+ waves promote triggered arrhythmias and we speculate that this occurs commonly in this patient population especially with their higher resting heart rates.

Both subcellular and intercellular discordant alternans developed during rapid pacing, but at much slower rates in CHF vs. normals. We have reported that both forms of intracellular and intercellular Ca2+ transient heterogeneities develop in myocytes of normal heart during rapid pacing. Intercellular discordant alternans occurs when neighboring myocytes develop alternans that is out-of-phase between cells. This highly heterogeneous Ca2+ cycling behavior establishes large Ca2+ gradients between myocytes and is likely to contribute to alterations in electrical activity at the cellular level. Subcellular alternans has been found in isolated atrial myocytes during rapid pacing, and in isolated ventricular myocytes under certain experimental conditions. Subcellular Ca2+ alternans establishes large intracellular Ca2+ gradients, which could increase the likelihood of Ca2+ waves and triggered activity. In the setting of CHF, where ultrastructural changes lead to dramatic deficiencies in E-C coupling, any additional influence that promotes Ca2+ waves and Ca2+ heterogeneities both within and...
between cells is likely to promote further electrical instabilities in the form of triggered and reentrant activity.

**Basis for triggered activity in CHF: Spontaneous and triggered Ca$^{2+}$ waves**

One of our most interesting observations was that Ca$^{2+}$ waves were not only spontaneous but were triggered during normal or rapid pacing in CHF. Spontaneous waves occurred in about 20% of myocytes and were not related to underlying cardiac activation. In contrast, over half of all sites studied showed a distinct population of myocytes with small transients that were interspersed with occasional large SR Ca$^{2+}$ release events (waves) that in some cases involved the entire cell and in others involved only parts of the cell. The distinguishing characteristic of triggered waves is that they occur during regular stimulation and not during the quiescent interval following cessation of pacing when spontaneous Ca waves arise.

Based on our results, we propose a scheme to explain the triggering requirement for these waves; t-tubule disruption and resulting defects in activation of SR Ca$^{2+}$ release allow little Ca$^{2+}$ to be released during normal stimulation. Ca$^{2+}$ accumulation then continues, leading to overload which is then responsible for Ca$^{2+}$ waves in response to very small local triggering events. In fact, we found subcellular releases in certain cell regions that do not propagate as waves and involve parts of the myocyte in a manner similar to subcellular alternans$^{8,25}$. However this behavior is activated, it appears to represent a novel form of defective Ca$^{2+}$ cycling in the setting of CHF that has not been described previously.
Rate dependent effects on defective Ca\textsuperscript{2+} cycling in CHF

We also found that defective Ca\textsuperscript{2+} handling in CHF is exaggerated as cardiac rate increases. Fractionation of both Ca\textsuperscript{2+} release and reuptake show a type of use-dependent suppression of Ca\textsuperscript{2+} cycling which progressively decreases contractile efficiency and could cause the negative force frequency relationship in myocardium from CHF patients\textsuperscript{26}. Fractionation of Ca\textsuperscript{2+} release has been reported in animal and human models of CHF, including SHRs, but without linking increased heart rate to pump failure and arrhythmias. Our results demonstrate that higher rates exaggerate the intracellular heterogeneities in SR Ca\textsuperscript{2+} release and reduce the efficacy of E-C coupling. We speculate that the increased SR Ca\textsuperscript{2+} load at higher heart rates can promote both spontaneous and triggered waves and therefore induce triggered arrhythmias. Thus, increased heart rate in the setting of CHF would both reduce contractile efficiency and promote triggered arrhythmias.

There is also increasing evidence that Ca\textsuperscript{2+} alternans may be responsible for T-wave alternans in ventricle\textsuperscript{22,27} since a large Ca\textsuperscript{2+} release from the SR would activate more inward NCX current and prolong APD than a small release. Thus endocardial cells with long transient durations develop both Ca\textsuperscript{2+} and APD alternans at slower rates than epicardial myocytes\textsuperscript{28,29} and myocytes in intact LV epicardium with long transients develop alternans at slower rates than adjoining myocytes with shorter transients\textsuperscript{8,30}. The basis for Ca\textsuperscript{2+} alternans is currently thought to arise from the steepness of the relationship between SR Ca\textsuperscript{2+} load and resulting release\textsuperscript{31}. Therefore, the shift of alternans sensitivity to slower heart rates in CHF could be a result of at least two processes that affect SR Ca\textsuperscript{2+} load, which unfortunately cannot be measured directly in
these experiments using a conventional rapid caffeine pulse. We have reported that the prolonged Ca^{2+} transients in CHF slows the recovery of Ca^{2+} release so that early activation would find less Ca^{2+} available for SR release, causing less release but allowing more time for recovery for the next beat in a Small-Large-Small-Large pattern. We speculate that, in addition to a prolongation of transient duration, the observed slowing in Ca^{2+} transient restitution could exaggerate this effect by further slowing SR recovery. Our results show that CHF is associated with both a prolongation of Ca^{2+} transients and a slowing in restitution, causing the development of an alternans pattern at slower than usual heart rates. The resulting APD alternans could therefore establish gradients of repolarization at slower heart rates in CHF, thus promoting reentrant arrhythmias.
Funding Sources

This work was supported in part by the Everett-O’Connor Trust.

Disclosures

None
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Wasserstrom JA. Pacing-induced heterogeneities in intracellular Ca2+ signaling, cardiac


Figure Legends

Figure 1. Linescan images showing intracellular Ca\textsuperscript{2+} transients of a multicellular site in control (WKY) and CHF (SHR) hearts. Panel A shows a transverse recording in WKY heart in which 15 myocytes are included in this recording site. Basal stimulation at BCL = 700msec evokes simultaneous activation of Ca\textsuperscript{2+} transients in all myocytes in which rapid release of stored Ca\textsuperscript{2+} is followed by slow reuptake. The average fluorescence intensity from all cells is shown above the image, giving a profile typical of intracellular Ca\textsuperscript{2+} transients. Cell borders are indicated by white dashed lines in the image and each cell is denoted by short thick lines to the left of each image. Intensity profiles of selected cells (A1-A4) are shown to the right. Panel B shows a recording from a site with 15 myocytes in an SHR heart with profiles of selected cells (B1-B5) at right.

Figure 2. WKY vs. SHR Ca\textsuperscript{2+} Transient Characteristics. Summary of characteristics of Ca\textsuperscript{2+} transient at basal pacing (BCL = 700msec).

Figure 3. WKY vs. SHR Heterogeneity Indices. Summary of heterogeneity indices for each of the Ca\textsuperscript{2+} transient characteristics during basal pacing.

Figure 4. Longitudinal recordings of intracellular Ca\textsuperscript{2+} transients in cell pairs of control and CHF hearts. Scan line was placed along the long axis of two cells in WKY (Panel A) and SHR (Panel B) hearts. Average fluorescence intensity profiles for each cell are placed above or below each image. BCL = 700msec.
Figure 5. Increasing heart rate promotes fractionation of intracellular Ca\textsuperscript{2+} transients in SHR. Panel A shows the linescan image of 10 myocytes in an SHR heart at BCL = 700 msec and during rapid pacing (BCL = 390 msec). Panel B shows an expanded scale image of two myocytes during basal pacing and Panel C shows transients during rapid pacing.

Figure 6. Development of rate-induced Ca\textsuperscript{2+} transient alternans in whole heart of normal and CHF animals. Panels A and B show intracellular Ca\textsuperscript{2+} transients at two pacing rates in a WKY heart. Panels C and D show transients in an SHR heart. Note that alternans develops at longer BCL in the SHR heart than in the WKY heart (340msec compared to 240msec). Panels E and F show sigmoidal curves of alternans ratio as a function of BCL for each myocyte.

Figure 7. WKY vs. SHR Ca\textsuperscript{2+} transient restitution. Summary of rates of Ca\textsuperscript{2+} transient restitution in individual myocytes in normal (WKY, Panel A) and CHF (SHR, Panel B). Restitution of SR Ca\textsuperscript{2+} release was measured as fractional recovery of the transient magnitude following a progressively shorter interval of the test beat (S2) compared to the last basal beat at BCL=700msec (S1). Each line represents the sigmoid fit to the fractional recovery (S2/S1) of each individual myocyte for all hearts.

Figure 8. Triggered waves in CHF. Panel A shows a longitudinal recording of a single SHR myocyte (BCL=400msec). The ECG is shown above and the average
fluorescence intensity profile is shown below the image. Red arrows indicate waves and asterisks indicate individual stimuli delivered to the whole heart. Note that rapid pacing was initiated before this portion of the image which shows Ca cycling and triggered waves during the steady state. Panel B shows recordings from two adjoining SHR myocytes in a transverse recording. Panel Bi shows linescan image at BCL = 700 msec and at BCL = 400 msec. Panel Bii shows the expanded image and profile of Cell 2 during rapid pacing (red box).
Figure 1

Representative Individual Cell Profiles

WKY

SHR

Normal release
Delayed release and peak
Abnormal release and wave

A

B

700 msec
50μ

WKY

SHR

Figure 1

Representative Individual Cell Profiles

WKY

SHR

Normal release
Delayed release and peak
Abnormal release and wave

A

B

700 msec
50μ
Figure 2

AMPLITUDE

RISE TIME

DECAY TIME

TOTAL Ca^{2+}

TD_{50}

TD_{80}

+ dF/dt

- dF/dt

ΔF/sec

ΔF/sec

WKY

SHR

**
Figure 3
Figure 4

A

Cell 1

WKY

Cell 2

50μ

B

Cell 1

SHR

Cell 2

700 msec
Figure 7

A

WKY
n = 60 myocytes
(4 sites in 3 hearts)

B

SHR
n = 72 myocytes
(4 sites in 3 hearts)
Multiple Defects in Intracellular Calcium Cycling in Intact Failing Rat Heart

Circ Heart Fail. published online March 30, 2009;
Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/early/2009/03/30/CIRCHEARTFAILURE.108.811539

Data Supplement (unedited) at:
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Male WKY and SHR rats (~21 months of age) were anesthetized with ketamine:xylazine (80:8mg/kg, IP) and the heart was removed and placed on a Langendorff apparatus for retrograde perfusion with a modified Tyrode’s solution. All animal use protocols were approved by the IACUC in accordance with NIH guidelines. Advanced stages of heart failure were demonstrated by high heart weight/body weight ratios (3.9±0.28 mg/gm in WKYs compared to 9.1±0.62 in SHRs, p<0.001, n=5), fluid in the lungs, dyspnea and greatly diminished activity levels in the SHR animals. The heart was then placed in an experimental chamber on the stage of a Zeiss LSM510 laser scanning confocal microscope (25±1°C). Fluo-4AM (15μM/L, Invitrogen) was added to the solution, which was then re-circulated for 30 min, and thereafter the heart was washed with normal solution. Cytochalasin-D (50μM/L, Sigma) was added to the solution to prevent contraction and re-circulation was again initiated. Hook platinum electrodes were inserted into the LV apex so that basal pacing at a basic cycle length (BCL) of 700msec could be interspersed with 10-second epochs of rapid pacing at different test CL. A section of LV mid-epicardial surface was scanned (488nm from a 25mW argon laser at ≤10% laser power). No recordings from the ventricular apex or base are included in this study. Once the contraction was abolished, a site is identified with multiple cells showing simultaneous activation in response to each stimulus. In addition, the brightness of the signal had to be sufficient to allow recordings with a good signal:noise ratio, even when fluorescence intensity was low (as in heart failure). Cell
borders were identified as regions of no fluorescence (indicating connective tissue bridges between cell bundles) or of constant high fluorescence intensity (indicating capillaries between myocytes). Cells could also be identified by the appearance of \( \text{Ca}^{2+} \) waves following rapid pacing in both WKYs and SHRs since the diffusion of \( \text{Ca}^{2+} \) waves between adjoining myocytes was a rare event. In most instances, 2-D images were recorded in order to define cell borders. Using all of these methods, it is quite unusual that we cannot definitively distinguish between myocytes during the course of a complete experiment (about 1-2%).

Ordinarily, the scan line was then placed transversely across the short axis of 10-25 myocytes and pacing protocols were initiated, although some recordings were made along the length of individual myocytes or cell pairs. Characteristics of \( \text{Ca}^{2+} \) transients were recorded during both basal and rapid pacing. The rate sensitivity of intracellular \( \text{Ca}^{2+} \) alternans magnitude was measured as alternans ratio \(^{8,9} \) (AR, 1 – small/large) at steady-state (10 sec) at each test BCL. Each epoch was followed by a 4-second pause before a return to basal pacing for 1 minute before the next test interval was initiated at a 10-msec shorter BCL. Pacing BCLs were tested in the range of 500-140msec or until 2:1 block or tachycardia occurred. Ventricular tachycardia (VT) was defined as a train of at least 5 spontaneous depolarizations following the test train.

Confocal fluorescence images were measured using Zeiss and ImageJ software. Fluorescence intensities for each cell were cut from the original images and intensity profiles were analyzed using MatLab and pCLAMP8 software. Mean data were compared using paired and unpaired Students \( t \)-tests or one-way ANOVA with appropriate secondary tests. All figures show mean+SEM for all characteristics of \( \text{Ca}^{2+} \)
transients. Measurements included peak amplitude of the transient, durations at 50% and 80% of recovery (TD50 and TD80), rise time from 10% to 90% of transient magnitude, decay time from 90% to 10% of peak, total Ca\(^{2+}\) released (the integral of basal transients), and the maximum rates of Ca\(^{2+}\) release and reuptake (+/- dF/dt). Magnitude of the transient was calculated from the ratio of fluorescence intensity at peak (F) to minimal intensity during the post-stimulation pause (F0). Heterogeneity indices (HI) were calculated to compare overall intercellular heterogeneity for each parameter within a site and were based on the standard deviation around the mean for that parameter\(^3\). Note that details of these methods have been published elsewhere\(^7\).

Restitution of SR Ca\(^{2+}\) release was measured by interpolating an extrasystole following the last beat at the basal BCL of 700msec. The first test interval was 600msec and subsequent intervals were progressively shortened until the interval was too short to activate Ca\(^{2+}\) release (block) or VT developed. The magnitude of the test beat is presented as a fraction of the preceding basal beat as fractional recovery. The rate of restitution was calculated for each myocyte and all cells from all hearts are graphed with fitted lines for clarity.

The use of paralytic agents to stop contraction could be problematic, since Cytochalasin-D has been shown to have direct effects on Ca\(^{2+}\) transients\(^{10}\). However, it has also been shown in rodent to be the least intrusive of the commonly used paralytic agents available\(^{11}\). Furthermore, we have found more recently that a lower concentration of Cytochalasin-D (30\(\mu\)M) in combination with blebbistatin (10-15\(\mu\)M) gives identical results, making us confident that the higher Cytochalasin-D concentration is having only modest effects on our results in this experimental setting. We have also
measured the effects of Cytochalasin-D on Ca$_{2+}$ transients in isolated rat myocytes and have found similar results to those reported previously, most notably a slight prolongation of time to peak and overall duration$^{10}$. However, despite these minor changes in Ca$_{2+}$ transients, it is likely that Cytochalasin-D would affect Ca$_{2+}$ transients in WKYs and SHRs in an identical manner; thus any effects of the Cytochalasin-D should be the same for both groups and are unlikely to explain the differences reported in this study.
LEGENDS FOR MOVIES

Supplemental Movie #1. Triggered waves in a region of LV epicardium in a 22 month SHR rat heart in CHF. Basal pacing at BCL=700msec is followed by a period of rest. Underlying small Ca$^{2+}$ transients during pacing upon which are superimposed bright waves that travel along the cell length. Note that waves cease immediately after pacing ends. Spontaneous waves begin several seconds after the end of the train. The same pattern of triggered waves superimposed upon very small transients begins again within a couple of beats when stimulation is resumed. Scale = 330µ per side.

Supplemental Movie #2. Lack of triggered waves during normal Ca$^{2+}$ cycling in a 22 month WKY rat heart. Basal pacing at BCL=700msec is followed by a 10-second train at BCL=360msec then a pause in stimulation. Basal pacing is resumed after the 4 second pause. There are no triggered waves, only spontaneous waves that develop late during the pause which is typical of normal rat heart.