The Mechanisms of Calcium Cycling and Action Potential Dynamics in Cardiac Alternans

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**Rationale:** Alternans is a risk factor for cardiac arrhythmia, including atrial fibrillation. At the cellular level, alternans manifests as beat-to-beat alternations in contraction, action potential duration (APD), and magnitude of the Ca\(^{2+}\) transient (CaT). Electromechanical and CaT alternans are highly correlated, however, it has remained controversial whether the primary cause of alternans is a disturbance of cellular Ca\(^{2+}\) signaling or electrical membrane properties.

**Objective:** To determine whether a primary failure of intracellular Ca\(^{2+}\) regulation or disturbances in membrane potential and AP regulation are responsible for the occurrence of alternans in atrial myocytes.

**Methods and Results:** Pacing-induced APD and CaT alternans were studied in single rabbit atrial and ventricular myocytes using combined [Ca\(^{2+}\)]\(_i\) and electrophysiological measurements. In current-clamp experiments, APD and CaT alternans strongly correlated in time and magnitude. CaT alternans was observed without alternation in L-type Ca\(^{2+}\) current, however, elimination of intracellular Ca\(^{2+}\) release abolished APD alternans, indicating that [Ca\(^{2+}\)]\(_i\) dynamics have a profound effect on the occurrence of CaT alternans. Trains of 2 distinctive voltage commands in form of APs recorded during large and small alternans CaTs were applied to voltage-clamped cells. CaT alternans was observed with and without alternation in the voltage command shape. During alternans AP-clamp large CaTs coincided with both long and short AP waveforms, indicating that CaT alternans develop irrespective of AP dynamics.

**Conclusions:** The primary mechanism underlying alternans in atrial cells, similarly to ventricular cells, resides in a disturbance of Ca\(^{2+}\) signaling, whereas APD alternans are a secondary consequence, mediated by Ca\(^{2+}\)-dependent AP modulation.

**Key Words:** action potentials • alternans • cardiac arrhythmias • calcium signaling • excitation-contraction coupling

Cardiac alternans is a recognized risk factor for cardiac arrhythmia, including atrial fibrillation, and sudden cardiac death. T-wave alternans in the ECG, corresponding to beat-to-beat alternations in ventricular repolarization, has become a prognostic tool for arrhythmia risk stratification and guidance of antiarrhythmic therapy. At the cellular level, cardiac alternans is defined as cyclic, beat-to-beat variations in contraction amplitude (mechanical alternans), action potential duration (APD or electrical alternans), and cytosolic Ca\(^{2+}\) transient (CaT) amplitude at constant stimulation frequency. A plethora of experimental conditions and interventions have been demonstrated to cause and modulate cardiac alternans, suggesting a multifactorial process. In cardiac myocytes, the beat-to-beat regulation of cytosolic calcium ([Ca\(^{2+}\)]\(_i\)) and membrane potential (V\(_m\)) is bidirectionally coupled and involves complex feedback mechanisms, often mediated by Ca\(^{2+}\)-dependent membrane conductances, that link these 2 parameters. It is generally agreed that this relationship represents a key causative factor for electromechanical and CaT alternans. The question whether V\(_m\)→[Ca\(^{2+}\)]\(_i\) or [Ca\(^{2+}\)]\(_i\)→V\(_m\) coupling is the primary underlying mechanism for alternans has been addressed almost exclusively in ventricular myocytes, but not in atria. It was suggested that at high stimulation rates V\(_m\) alternations are determined by APD restitution and is an underlying cause for the development of alternans (V\(_m\)→[Ca\(^{2+}\)]\(_i\)). APD restitution refers to the APD dependence on the preceding diastolic interval and, if this relationship is steep enough, self-sustaining oscillations of APD can occur. In this case, the time-dependent recovery of ion channels from inactivation, in particular, recovery of L-type Ca\(^{2+}\) channels (LCC), has been hypothesized as a causative factor for the generation of alternans. Conversely, other studies demonstrated a poor relationship between experimentally
determined APD restitution kinetics and occurrence of alternans, and it was suggested that disturbances in beat-to-beat Ca\textsuperscript{2+} cycling constitute the main cause of cardiac alternans ([Ca\textsuperscript{2+}"	extsubscript{i}]→[Ca\textsuperscript{2+}"	extsubscript{i}]→V\textsubscript{m}). In this case, V\textsubscript{m} is determined by the effect and consequences of [Ca\textsuperscript{2+}"	extsubscript{i}] dynamics, Ca\textsuperscript{2+} fluxes, and Ca\textsuperscript{2+}-dependent ion currents and transporters.

In light of these unresolved issues, we set out to determine the detailed effects of AP morphology on the occurrence of CaT alternans in atrial myocytes where V\textsubscript{m}→[Ca\textsuperscript{2+}"	extsubscript{i}] coupling has not been investigated systematically, despite the fact that atrial fibrillation is the most common cardiac arrhythmia and has been causally linked to alternans. Although it is anticipated that mechanisms of alternans in atrial and ventricular cells share similarities, significant differences are also expected. Atrial and ventricular cells exhibit distinct differences in Ca\textsuperscript{2+} cycling during excitation–contraction coupling because of the lack of or poor development and irregular organization of transverse (t)-tubules and higher sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) activity in the atria. Computer simulations using cell models with and without t-tubules have predicted significant differences in possible alternans mechanisms but also exhibit important differences, and the role of V\textsubscript{m}→[Ca\textsuperscript{2+}"	extsubscript{i}] coupling for the generation of electromechanical alternans is likely secondary to the disturbances in Ca\textsuperscript{2+} signaling.

**Methods**

**Cell Isolation**

Ventricular and atrial myocytes were enzymatically isolated from New Zealand White rabbits via Langendorff perfusion. All procedures and protocols involving animals conform to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee.

**Electrophysiological Measurements**

Electrophysiological recordings were made in the ruptured whole-cell patch-clamp configuration using an Axopatch 200A patch-clamp amplifier. For AP measurements, the whole-cell current-clamp mode was used. For L-type Ca\textsuperscript{2+} channel current (I\textsubscript{LCC}) measurements, membrane currents were elicited by 100-ms depolarization steps to 0 mV from a holding potential of −50 mV. For AP-clamp experiments, voltage commands were derived from previously recorded atrial or ventricular AP-waveforms.

**Cytosolic [Ca\textsuperscript{2+}"	extsubscript{i}] Measurements**

Simultaneously with electrophysiological recordings, cytosolic [Ca\textsuperscript{2+}"	extsubscript{i}] was recorded using Fluo-4 or Indo-1 pentapotassium salts added to the patch pipette solution. Fluo-4 was excited with a 488-nm argon ion laser and recorded at 515 nm. Fluo-4 emission signals (F) were background subtracted and normalized to baseline fluorescence (F\textsubscript{0}), and changes of [Ca\textsuperscript{2+}"	extsubscript{i}] are presented as changes of ratio of background subtracted Indo-1 fluorescence emission at 410 nm (F\textsubscript{410}) and 485 nm (F\textsubscript{485}). Signals were background subtracted and changes of [Ca\textsuperscript{2+}"	extsubscript{i}] are expressed as changes of ratio of background subtracted Indo-1 fluorescence emission at 410 and 485 nm (R=I\textsubscript{F410}/I\textsubscript{F485}). CaT alternans was induced by increasing the pacing frequency until stable CaT alternans was observed. The degree of CaT alternans was quantified as the alternans ratio (AR), defined as AR=1−[CaT\textsubscript{small}/CaT\textsubscript{large}], where CaT\textsubscript{small} and CaT\textsubscript{large} are the small- and large-amplitude CaTs from a pair of alternating CaTs. All experiments were performed at room temperature (20–22°C).

**Data Analysis and Presentation**

Results are presented as individual observations or as means±SEM of n cells. Statistical significance was evaluated using Student t test and differences were considered significant at P<0.05.

**Results**

**Beat-to-Beat AP Morphology During CaT Alternans**

Alternans was induced by incrementally increasing the rate of electric pacing of current-clamped myocytes. Increasing the pacing rate eventually led to the simultaneous onset of beat-to-beat alternations in both CaT amplitude and AP morphology (the threshold frequencies where alternans was induced are summarized in Online Figure 1). Figure 1 shows examples of simultaneous recordings of APs and CaTs from single atrial (Figure 1A) and ventricular (Figure 1C) cells. In all cells tested, the onset of APD alternans coincided in time with the
onset of CaT alternans. Figure 1B and 1D shows superimposed APs recorded simultaneously with large (APCaT_Large) and small (APCaT_Small) amplitude CaTs from atrial and ventricular cells. The AP waveforms shown here reflect an average of 3 consecutive AP recordings. In both atrial and ventricular cells, APCaT_Small exhibited a more pronounced plateau phase followed by steeper repolarization compared with AP CaT_Large. APD at 30%, 50%, and 90% repolarization (APD30, APD50, and APD90) for AP CaT_Large and AP CaT_Small were compared. AP CaT_Small increased in duration at APD30 and shortened at APD90 level. Although changes in APD50 were small, the majority of atrial and ventricular cells exhibited a slightly wider APD50 during the small amplitude CaT. In atrial myocytes, CaTs alternated with an average CaT AR of 0.64±0.04, APD30CaT_Small and APD50CaT_Small increased by 86±11% and 26±8%, respectively, whereas APD90CaT_Small decreased by 12±5% (n=14). In ventricular myocytes, CaTs alternated with an average AR of 0.55±0.09. APD30CaT_Small and APD50CaT_Small increased by 32±6% and 3±3%, respectively, and APD90CaT_Small shortened by 8±3% (n=10).

In both atrial and ventricular myocytes, changes in APD correlated with the CaT AR, indicating a tight link between APD and CaT alternans. For each cell tested, AP CaT_Small/APD CaT_Large ratios were plotted versus AR of the CaTs and fitted with a linear regression function to help categorize the data. Figure 2 shows that for APD30 and APD50, AP CaT_Small/APD CaT_Large ratios increased with increasing AR, whereas for APD90 the AP CaT_Small/APD CaT_Large ratio slightly decreased in both atrial (Figure 2A) and ventricular (Figure 2C) cells (data derived from the same cells as shown in Figure 1). Linear regression slopes for all individual cells, as well as the averages for each data set are presented in Figure 2B and 2D.

In conclusion, the onset and progression of APD alternans in cardiac myocytes correlated with the alternation in [Ca2+]i, time and magnitude. AP CaT_Small recorded during a small amplitude alternans CaT exhibited a more prominent plateau phase and showed faster repolarization resulting in an increase of APD30 and APD50, and a shortening of APD90. The most pronounced beat-to-beat alternation was observed at APD30 level in both atrial and ventricular cells. Thus, although qualitative changes in APDs at different degrees of repolarization were the same in atrial and ventricular cells, overall the beat-to-beat differences in APD were clearly more pronounced in atrial myocytes.

Ca2+ Transients are Not Driven by the Changes in AP Morphology

To gain further insight whether cardiac alternans is driven by disturbances of electrical membrane properties and alternating changes in inherent AP characteristics (Vm→[Ca2+]i coupling) or is caused by a primary defect in intracellular Ca2+ cycling ([Ca2+]i→Vm coupling), we conducted several series of AP-clamp experiments combined with simultaneous measurements of [Ca2+]i. For this purpose, atrial and ventricular myocytes were voltage-clamped with a voltage command in the form of APs that were previously recorded in current-clamp mode from the respective cell type exhibiting CaT alternans. AP-clamp voltage protocols were then constructed as a series of AP-waves consisting (1) exclusively of APs recorded during a large amplitude alternans CaT (AP CaT_Large protocol); (2) exclusively of AP CaT_Small recorded during a small amplitude alternans CaT (AP CaT_Small protocol); and (3) of alternating AP (AP CaT_Large→AP CaT_Small protocol, also referred to here as alternans AP-clamp).
Atrial and ventricular AP\textsubscript{CaT\_Small} and AP\textsubscript{CaT\_Large} morphologies are discussed in Figure 1.

In the first set of experiments, cells were paced by a series of AP-waveform commands of the same shape (AP\textsubscript{CaT\_Large}–AP\textsubscript{CaT\_Large} and AP\textsubscript{CaT\_Small}–AP\textsubscript{CaT\_Small} pacing protocols), and under these conditions membrane voltage was identical from beat-to-beat. Both AP\textsubscript{CaT\_Large}–AP\textsubscript{CaT\_Large} and AP\textsubscript{CaT\_Small}–AP\textsubscript{CaT\_Small} pacing protocols induced CaT alternans in atrial (n=9; Figure 3A and 3B) and ventricular myocytes (n=10; Figure 3C and 3D). The pacing rates required to induce CaT alternans with these protocols varied from 1 to 1.6 Hz (see also Online Figure I for average alternans induction thresholds) and thus, were in a similar range as in current-clamp experiments (Figure 1). These data indicate that beat-to-beat alternation in the intracellular Ca\textsuperscript{2+} release does not require APD alternans and are consistent with previous findings in isolated ventricular myocytes.\textsuperscript{29,30} Here, it is demonstrated that atrial cells exhibit similar behavior and CaT alternans can develop independently of membrane voltage and in the absence of APD alternans.
In the next set of experiments, a true alternans AP-clamp (AP\textsubscript{CaT\_Large} – AP\textsubscript{CaT\_Small}) protocol was applied. When pacing frequency was reduced below alternans thresholds, CaT alternans disappeared, despite the alternating AP voltage commands (Figure 4A and 4B) in both atrial and ventricular myocytes. At higher pacing frequencies (ranging from 1 to 1.8 Hz in atrial and from 0.8 to 1.6 Hz in ventricular myocytes; see also Online Figure I), CaT alternans could be elicited reliably (Figure 4C and 4D). We analyzed how the amplitude of the CaTs correlated with AP\textsubscript{CaT\_Large} or AP\textsubscript{CaT\_Small} voltage pacing commands during the alternans AP-clamp protocol. For this discussion, we refer to the situation where the AP\textsubscript{CaT\_Large} elicited a large amplitude CaT and the AP\textsubscript{CaT\_Small} triggered a small amplitude CaT as in-phase, whereas the coincidence of AP\textsubscript{CaT\_Small} and large amplitude CaT (and vice versa) is termed out-of-phase. Although out-of-phase alternans was observed in both atrial and ventricular myocytes, atrial cells revealed a higher propensity of in-phase alternans compared with ventricular cells (Figure 4E). In ventricular myocytes, of 15 cells tested, in 4 cells CaT alternans was exclusively in-phase, 9 cells exhibited both in-phase and out-of-phase alternans, and in 2 cells only out-of-phase alternans was observed, thus no clear tendency in favor of in-phase versus out-of-phase alternans could be identified. In contrast, in 8 atrial cells, only in-phase CaT alternans was observed and 6 cells exhibited both in-phase and out-of-phase alternans, whereas no atrial cells that would develop exclusively out-of-phase alternans were observed (total n=14 cells).

In summary, our data show that under voltage-clamp conditions, CaT alternans develop in the absence or presence of APD (or electric) alternans. Furthermore, CaT alternans can develop irrespective of AP dynamics and can be in-phase or out-of-phase as defined above. In addition, CaT alternans can even be absent, despite APD alternans. These data are strong indication that CaT alternans results from intrinsic properties of intracellular Ca\textsuperscript{2+} handling, and changes in APD alone are not sufficient to cause CaT alternans. Thus, the results strongly support a [Ca\textsuperscript{2+}]\textsubscript{i}→V\textsubscript{m} coupling paradigm for the genesis of electromechanical alternans. Furthermore, at first approximation atrial and ventricular cells behaved similarly during alternans AP-clamp experiments: both cell types revealed in-phase and out-of-phase alternans, however, there were subtle differences in the relative frequency and prevalence of the 2 forms of alternans.

Changes in L-Type Ca\textsuperscript{2+} Current are Not Required for CaT Alternans

It has been suggested that APD alternans and alternations in AP morphology might reflect beat-to-beat changes in I\textsubscript{LCC}. Because we observed the largest beat-to-beat APD variability at APD30, that is, at a voltage near the plateau phase of the AP (Figure 1) where I\textsubscript{LCC} contributes to AP morphology, the hypothesis was tested whether beat-to-beat changes in I\textsubscript{LCC}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Ca\textsuperscript{2+} transient (CaT) alternans induced independently of cell voltage. At lower pacing frequencies, CaT alternans was not observed in atrial (A) and ventricular (B) cells paced with the AP\textsubscript{CaT\_Large} – AP\textsubscript{CaT\_Small} voltage protocol referred to as alternans action potential (AP)-clamp (bottom trace). During alternans AP-clamp large CaTs were observed with AP\textsubscript{CaT\_Large} (in-phase, top trace) or with AP\textsubscript{CaT\_Small} (out-of-phase, middle trace) voltage command (bottom). [Ca\textsuperscript{2+}]\textsubscript{i} traces were recorded from the same atrial (C) and ventricular (D) myocyte. E, Total number of atrial and ventricular myocytes exhibiting CaT alternans that were exclusively in-phase (white), exclusively out-of-phase (black), or revealed both in-phase and out-of-phase alternans (gray).}
\end{figure}
are required for the induction of CaT alternans. For reasons of comparison between atrial and ventricular cells, identical experiments were performed in both cell types. Figure 5A shows simultaneous recordings of $I_{\text{LCC}}$ and CaTs in a voltage-clamped ventricular myocyte. $I_{\text{LCC}}$ was activated by a 100-ms voltage step from a holding potential of −50 to 0 mV. Peak $I_{\text{LCC}}$ was measured as the difference between the peak of the inward current and the current level at the end of 100-ms pulse. The stimulation rate varied from 1.3 to 2 Hz to initiate CaT alternans, and the average threshold frequency was slightly higher than in current- and AP-clamp experiments (Online Figure I). In the example shown (Figure 5A), CaT alternans with an AR of 0.29 was observed at the stimulation rate of 1.3 Hz. In both cell types, CaT alternans were induced by the conventional square pulse voltage protocols and were observed without apparent alternation in peak $I_{\text{LCC}}$. The average ratios of peak $I_{\text{LCC}}$ (Figure 5B) measured during a small ($I_{\text{LCC,S}}$) and a large ($I_{\text{LCC,L}}$) amplitude CaT were 0.99±0.01 and 0.97±0.05 in atrial (n=4) and ventricular (n=5) cells, respectively. In contrast, the simultaneously recorded CaT ARs were 0.35±0.06 and 0.42±0.12. $I_{\text{LCC}}$ recorded simultaneously with the small-amplitude CaT exhibited slightly slower inactivation, presumably because of reduced Ca$^{2+}$-dependent inactivation (CDI).

In summary, with respect to the relationship between $I_{\text{LCC}}$ and CaT alternans, these experiments indicate that (1) CaT alternans can be induced without significant beat-to-beat variations in peak $I_{\text{LCC}}$, (2) CaT alternans can be elicited with a conventional square pulse voltage protocols, an observation that provides additional support for the concept that $V_m \rightarrow [\text{Ca}^{2+}]_i$ coupling is not a causative factor for cardiac alternans, and (3) because CDI of $I_{\text{LCC}}$ is reduced during the small amplitude CaT, $I_{\text{LCC}}$ is likely to contribute to the prolongation of APD30 observed during the small amplitude CaT (Figure 1), indicating the importance of feedback mechanisms for the regulation of $V_m \leftrightarrow [\text{Ca}^{2+}]_i$ coupling.

### Inhibition of SR Ca$^{2+}$ Release Abolishes APD Alternans

To further test the hypothesis that APD alternans is driven by alternations in [Ca$^{2+}$]$_i$, intracellular Ca$^{2+}$ release was inhibited by application of 10 μmol/L ryanodine. Figure 6A shows APs and simultaneously recorded CaTs before and after application of ryanodine. The average CaT AR before application of ryanodine was 0.72±0.06 (n=4). The data summarized in Figure 6B show that the application of ryanodine eliminated APD alternans and significantly reduced APD30$_{\text{CaT_Small}}$/APD30$_{\text{CaT_Large}}$ ratios from 1.98±0.30 observed in control to 1.11±0.04 (P<0.01) and APD50$_{\text{CaT_Small}}$/APD50$_{\text{CaT_Large}}$ from 1.25±0.12 to 1.08±0.03 (P<0.05). APD90$_{\text{CaT_Small}}$/APD90$_{\text{CaT_Large}}$ ratios were 1.03±0.07 and 1.03±0.04 before and after application of ryanodine, respectively (absolute APD30, APD50, and APD90 data are presented in Online Figure II). Similar results were observed in ventricular cells (data not shown) and are in line with previous observations that inhibition of SR release abolishes APD alternans in ventricle of the whole heart and in single ventricular myocytes.

In summary, the results demonstrate that suppression of SR Ca$^{2+}$ release abolished beat-to-beat alternation in APD, further supporting the notion that instabilities in intracellular Ca$^{2+}$ handling represent a key factor in the development of APD alternans.

### Discussion

In this study, we investigated the interplay between electrical membrane properties and the beat-to-beat [Ca$^{2+}$]$_i$ dynamics that result in the occurrence of cardiac alternans in atrial and ventricular myocytes. The key findings are (1) current-clamp experiments revealed that APD and CaT alternans strongly correlated in time and magnitude in both atrial and ventricular cells, but the beat-to-beat difference in APD was significantly larger in atrial cells, (2) CaT alternans was observed without changes in peak $I_{\text{LCC}}$, however, during the large amplitude CaT CDI of $I_{\text{LCC}}$ was more pronounced, (3) voltage-clamp experiments using AP-clamp protocols revealed that CaT alternans occurred irrespective of whether cells were stimulated with

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**Figure 5.** Simultaneous recordings of L-type Ca$^{2+}$ current and Ca$^{2+}$ transient (CaT) alternans. 
A. Simultaneous recordings of L-type Ca$^{2+}$ channel current ($I_{\text{LCC}}$) and CaTs from a voltage-clamped ventricular myocyte during CaT alternans. $I_{\text{LCC}}$ was elicited with 100-ms depolarization steps from a holding potential of −50 to 0 mV (bottom). B. Summary data for simultaneously recorded $I_{\text{LCC,S}}$/AR$_{I_{\text{LCC,S}}}$ and CaTs alternans ratio (AR) in atrial (n=4) and ventricular (n=5) myocytes. $I_{\text{LCC,S}}$: peak L-type Ca$^{2+}$ current recorded with small amplitude Ca$^{2+}$ transient; $I_{\text{LCC,L}}$: peak current recorded simultaneously with large CaT. C. Current-voltage relationship of $I_{\text{LCC}}$ recorded from ventricular myocytes (n=9). L indicates large amplitude CaT, and S, small amplitude CaT.
Role of Bidirectional Coupling of [Ca\textsuperscript{2+}] and V\textsubscript{m} in the Development of Cardiac Alternans

The multitude of experimental conditions and interventions that cause and modulate Ca\textsuperscript{2+} and electro-mechanical alternans have undoubtedly demonstrated that cardiac alternans is a multifactorial process. Although it is well accepted that electrical (APD), mechanical, and CaT alternans strongly correlate,\textsuperscript{23,53,54} a comprehensive mechanism that can explain and predict the occurrence of cardiac alternans has not been fully established to date, although recently progress was made toward a unifying overarching conceptual framework for alternans in ventricular myocytes.\textsuperscript{14,55-57} In addition, in the process of a unifying theory of cardiac alternans invaluable insight has come from computational models of cardiac Ca\textsuperscript{2+} signaling and ion current activity during excitation-contraction coupling and APD alternans.\textsuperscript{9,10,14} The key concept behind these computational models is the paradigm that the beat-to-beat regulation of V\textsubscript{m} and [Ca\textsuperscript{2+}] are bidirectionally coupled, and feedback mechanisms, often mediated by Ca\textsuperscript{2+} play a crucial role. Bidirectional coupling of V\textsubscript{m} and [Ca\textsuperscript{2+}] is defined by the facts that (1) V\textsubscript{m} directly determines the activity of Ca\textsuperscript{2+} handling mechanisms that are voltage-dependent (V\textsubscript{m}→[Ca\textsuperscript{2+}] coupling), whereas (2) [Ca\textsuperscript{2+}]→V\textsubscript{m} coupling is determined by the effect of Ca\textsuperscript{2+}-dependent ion currents and transporters. In current-clamp experiments (Figure 1), we confirmed that Ca\textsuperscript{2+} and APD alternans were closely coupled in both atrial and ventricular myocytes. Alternans developed at pacing rates >1 Hz (Online Figure I), there was no temporal dispersion in the onset of CaT and APD alternans, and the degree of APD and CaT alternans correlated closely (Figure 2). Furthermore, we investigated which direction of coupling—V\textsubscript{m}→[Ca\textsuperscript{2+}] or [Ca\textsuperscript{2+}]→V\textsubscript{m}—plays the key role in the development of cardiac alternans.

V\textsubscript{m}→[Ca\textsuperscript{2+}] Coupling and Cardiac Alternans

Previous studies have suggested a primary role of [Ca\textsuperscript{2+}]→V\textsubscript{m} in ventricular cells,\textsuperscript{22,27,58} however, for atrial myocytes the data are scarce. Thus, we aimed to collect direct experimental evidence in favor of or against V\textsubscript{m}→[Ca\textsuperscript{2+}] coupling in atrial cells and compared with data obtained from ventricular myocytes. We designed an experimental approach where V\textsubscript{m} was treated as an independent variable and [Ca\textsuperscript{2+}] was viewed as the dependent parameter controlled by V\textsubscript{m}. Application of series of same-shape AP waveforms (AP\textsubscript{C,Gate} (Figure 3A and 3C) or AP\textsubscript{C,Gat,Small} (Figure 3B and 3D) resulted in CaT alternans in both atrial and ventricular myocytes, that is, CaT alternans developed, provided the pacing rate was sufficiently high, irrespective of AP duration and shape. In a next set of experiments, we applied a voltage-clamp protocol that consisted of APs with alternating duration, termed as alternans AP-clamp. As shown in Figure 4 with the alternans AP-clamp protocol, 3 qualitatively different Ca\textsuperscript{2+} responses could be elicited, such as (1) no CaT alternans, (2) in-phase alternans where the large CaT was observed with the AP\textsubscript{C,Gate} command, and (3) out-of-phase alternans where the large Ca\textsuperscript{2+} signal coincided with AP\textsubscript{C,Gat,Small}. The AP voltage-clamp experiments clearly demonstrate that CaT alternans can develop in the absence or presence of APD alternans. Furthermore, the identical alternans AP-clamp protocol could elicit in-phase and out-of-phase alternans in the same individual cell, arguing against the possibility that these results could be attributed to variability between cells. These results strongly argue against V\textsubscript{m}→[Ca\textsuperscript{2+}] coupling being a dominant causative factor for the generation of CaT alternans in both atrial and ventricular myocytes.

[Ca\textsuperscript{2+}]→V\textsubscript{m} Coupling and Cardiac Alternans

Additional support for the paradigm that [Ca\textsuperscript{2+}]→V\textsubscript{m} coupling as the primary cause of atrial alternans was obtained from the experiments shown in Figure 6. In current-clamp experiments combined with simultaneous [Ca\textsuperscript{2+}] measurements, atrial myocytes that revealed stable APD and CaT alternans were exposed to ryanodine to eliminate SR Ca\textsuperscript{2+} release without affecting Ca\textsuperscript{2+} entry via I\textsubscript{LCl}. Ryanodine treatment completely abolished SR Ca\textsuperscript{2+} release and subsequently CaT alternans, and at the same time stabilized APD and AP morphology. The observation of elimination of alternans by ryanodine in atrial myocytes is consistent with earlier finding obtained mainly\textsuperscript{59} in ventricular tissue.\textsuperscript{22,51,53} In addition, we have shown previously that beat-to-beat restitution properties of SR Ca\textsuperscript{2+} release,\textsuperscript{28} as well as the interplay between mitochondria,\textsuperscript{60,62} cellular metabolism,\textsuperscript{54,62} and Ca\textsuperscript{2+} signaling influence the likelihood of cardiac alternans, providing altogether strong evidence that the primary cause of cardiac...
alternans lies in disturbances of cellular beat-to-beat Ca$^{2+}$
signaling.

**Putative Mechanisms of CaT Alternans**

Although evidence in favor of [Ca$^{2+}$]$_{i}$→Vm coupling as the primary cause of alternans is growing, it is still unresolved, which disturbances of Ca$^{2+}$ regulatory mechanisms and processes are ultimately responsible for CaT alternans. Here, we summarize, in the context of our new experimental data, putative mechanisms.

**SR Ca$^{2+}$ Load and CaT Alternans**

Under steady-state conditions, SR Ca$^{2+}$ release and uptake by SERCA are well balanced, resulting in little beat-to-beat variation in diastolic [Ca$^{2+}$]$_{i}$SR. If the balance between Ca$^{2+}$ uptake and release is disturbed beat-to-beat alternation in diastolic [Ca$^{2+}$]$_{i}$SR might occur. Because oscillations in diastolic SR Ca$^{2+}$ load have been observed, it did not come as a surprise that beat-to-beat instabilities in [Ca$^{2+}$]$_{i}$SR were proposed as an underlying mechanism of alternans. Because of the steep SR load-Ca$^{2+}$ release relationship a higher SR load is expected to lead to a larger CaT and vice versa. However, contrary to these observations, others have reported CaT alternans without significant change in end-diastolic SR load in single myocytes and intact heart indicating that alternation in diastolic [Ca$^{2+}$]$_{i}$SR is not a required condition for CaT alternans to occur. Particularly in atrial myocytes, the absence of diastolic [Ca$^{2+}$]$_{i}$SR alternans during cytosolic alternans was a common observation and might be related to the lower expression of the endogenous SERCA inhibitor phospholamban and a higher activity of SERCA and capacity to completely refill the SR on every beat.

**I_{LCC} and CaT Alternans**

Under the concept of bidirectional coupling of [Ca$^{2+}$]$_{i}$ and V$_{m}$, I$_{LCC}$ plays a unique role: I$_{LCC}$ represents the critical trigger for Ca$^{2+}$-induced Ca$^{2+}$ release, and SR Ca$^{2+}$ release (and ultimately the amount of Ca$^{2+}$ that becomes available for contraction) is graded with I$_{LCC}$. The activity of I$_{LCC}$ is controlled by voltage and Ca$^{2+}$ itself: activation of I$_{LCC}$ is voltage-dependent, and inactivation of the currents is subject to a complex voltage- and Ca$^{2+}$-dependence. Thus, it comes as no surprise that beat-to-beat alternations of I$_{LCC}$ have been proposed as a causative factor of CaT alternans. A potential mechanism would entail incomplete time-dependent recovery from inactivation of I$_{LCC}$ and a reduced I$_{LCC}$ was indeed shown to increase susceptibility to CaT alternans. This hypothesis, however, would have to reconcile the observation that peak I$_{LCC}$ is unchanged during alternans in both ventricular and atrial myocytes (Figure 5B).

**Alternans in Atrial and Ventricular Myocytes**

The main aim of this study was to establish whether V$_{m}$→[Ca$^{2+}$]$_{i}$ or [Ca$^{2+}$]$_{i}$→Vm coupling is the primary mechanism for alternans in atrial cells. To date, this question has been addressed primarily in ventricular tissue and to a much lesser extent in atrial myocytes. To allow for reliable comparison between mechanisms of alternans in atrium and ventricle, we performed analogous experiments under the same conditions in both types of cardiomyocytes. Although it is anticipated that mechanisms of alternans in atrial and ventricular cells will share similarities, significant differences are also expected. The main structural difference between ventricular and atrial myocytes is that atrial cells lack or have only a poorly or irregularly developed t-tubule system resulting in unique Ca$^{2+}$ cycling features during excitation–contraction coupling. In atrial myocytes, lacking t-tubules LCCs are located only in the periphery of the cell and thus, membrane depolarization induced Ca$^{2+}$ release first occurs in subsarcolemmal regions and then propagates to the center of the cell. Computer simulations using cell models with and without t-tubules have predicted significant differences in possible alternans mechanisms. The cardiac cell models lacking t-tubules exhibited higher likelihood to develop CaT alternans and pointed toward the role of Ca$^{2+}$ diffusion, inhomogeneities in [Ca$^{2+}$]$_{i}$, and RyR refractoriness in the process. This is consistent with...
Spatial and temporal inhomogeneities in [Ca\textsuperscript{2+}], during atrial alternans found experimentally. As we have shown previously, during alternans, especially the small amplitude CaT was spatially inhomogeneous, there are intracellular gradients of the AR, and subcellular regions can alternate out-of-phase. The latter is of particular interest because the border between subcellular regions alternating out-of-phase is highly susceptible to spontaneous arrhythmogenic Ca\textsuperscript{2+} release and represent a frequent site of origin of spontaneous Ca\textsuperscript{2+} waves. Another difference in Ca\textsuperscript{2+} handling between atrial and ventricular cells is the lower expression of phospholamban that leads to higher SERCA activity in the atria. The beat-to-beat fluctuation in SR load was proposed as a possible cause of CaT alternans. It is conceivable that the lower SERCA activity in ventricle may contribute to CaT alternans at increased pacing frequencies because of incomplete filling of the SR, whereas a more rapid filling is consistent with the observation that in atrial cells, end-diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} typically did not alternate during CaT alternans.

In this study, irrespective of whether current-clamp experiments were conducted in atrial or ventricular myocytes, CaT and APD alternans coincided closely and the degree of CaT and APD alternans correlated well (Figures 1 and 2). In both cell types, the biggest beat-to-beat difference in APD was observed at APD30 (corresponding essentially to the plateau phase of the AP). Nonetheless, there were subtle differences between atrial and ventricular myocytes and measured relative beat-to-beat differences in APD were larger in atrial myocytes. In addition, a slightly higher pacing frequency was needed to induce alternans in atrial myocytes (Online Figure I). The basis of this difference between atrial and ventricular cells remains unclear. It is probable that degree of APD alternans is determined by the distinctive set of ion channels and transporters typical for each cell type. For example, ventricle and atrium differ in the activity of Ca\textsuperscript{2+}-activated small conductance K\textsuperscript{+}, whereas acetylcholine-activated and ultrarapid rectifier K\textsuperscript{+} channels are expressed exclusively in the atria. However, duration of the atrial AP is significantly shorter and this per se can result in the bigger relative change of APD, even if atrial and ventricular cells share similar Ca\textsuperscript{2+}-dependent mechanisms leading to APD alternation.

To recreate V\textsubscript{m} changes occurring during APD alternans, voltage-clamped cardiomyocytes were paced with cell type-specific alternans AP-clamp voltage protocols. These experiments demonstrated that CaT alternans can be in-phase or out-of-phase in both atrial and ventricular myocytes (Figure 4E), that is, a large amplitude CaT can coincide with AP\textsubscript{Ca,T,Large} or with AP\textsubscript{Ca,T,Small}. Most atrial cells showed exclusively in-phase alternans, and no atrial cell showed exclusively out-of-phase alternans. In contrast, the majority of ventricular cells revealed both in-phase and out-of-phase alternans, and there was a fraction of ventricular cells that showed exclusively out-of-phase alternans. This finding suggests that V\textsubscript{m}→[Ca\textsuperscript{2+}]\textsubscript{SR} coupling might play a more prominent role in atrial than in ventricular cells. In contrast, there seems to be a higher degree of independence of CaT alternans from AP morphology in ventricular cells. Atrial cells, however, have a bigger relative beat-to-beat difference in APD. It is expected that the alternating voltage will affect activity of LCC and Ca\textsuperscript{2+} removal by Na/Ca exchange, and thus will significantly modulate stability and severity of CaT alternans. In addition, the shape of the AP was shown to modulate SR Ca\textsuperscript{2+} load. Therefore, the bigger relative alternation in APD might make atria more susceptible to alternans and arrhythmias.

**Summary and Conclusions**

With the combined use of the patch-clamp technique and intracellular Ca\textsuperscript{2+} indicator dyes, we demonstrate that cardiac alternans arise from disturbances of intracellular Ca\textsuperscript{2+} signaling and that the concomitant characteristic changes in AP morphology are secondary to changes in [Ca\textsuperscript{2+}], and mediated through mechanisms discussed in details. The causes of Ca\textsuperscript{2+} and electromechanical alternans are multiple and reflect the inherently complex beat-to-beat regulation of [Ca\textsuperscript{2+}], and V\textsubscript{m} referred to here as the bidirectional coupling of [Ca\textsuperscript{2+}], and V\textsubscript{m}. It has become increasingly clear that cardiac alternans are predominantly driven by [Ca\textsuperscript{2+}]→V\textsubscript{m} coupling, however, through complex feedback mechanisms V\textsubscript{m}→[Ca\textsuperscript{2+}] coupling plays a modulatory role in the regulation of alternans. In our study, striking similarities between atrial and ventricular cells with respect to the importance of [Ca\textsuperscript{2+}]→V\textsubscript{m} coupling were found. However, larger alternations of APD implies a potentially more prominent role of V\textsubscript{m}→[Ca\textsuperscript{2+}] coupling in modulation of alternans in the atria.

**Sources of Funding**

This work was supported by National Institutes of Health grants HL62231, HL80101, and HL101235 and the Leducq Foundation (to L.A. Blatter).

**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is Known?**

- Cardiac alternans, described as beat-to-beat variation in contraction, action potential (AP) morphology, and intracellular Ca2+ release, is a recognized risk factor for life-threatening arrhythmias.
- The interplay between intracellular Ca2+ handling, membrane potential and AP morphology underlies electrophysiological alternans, however, a mechanistic understanding of the alternans phenomenon is still incomplete.
- Previous studies investigating mechanisms of Ca2+ alternans have been performed mostly in ventricular but not in atrial tissue.

**What New Information Does This Article Contribute?**

- Beat-to-beat changes in action potential duration and morphology alone are not sufficient to induce Ca2+ transient alternans in atrial and ventricular myocytes.
- AP morphology alternans arise from disturbances of intracellular Ca2+ signaling.
- The underlying mechanisms of alternans reveal similarities in atrial and ventricular myocytes, however, beat-to-beat changes in action potential duration and voltage-dependent modulation of Ca2+ transient alternans are more pronounced in the atrium.

Cardiac alternans is causatively linked to ventricular arrhythmias, atrial fibrillation, and sudden cardiac death. The mechanisms of alternans are complex, and the bidirectional coupling between beat-to-beat cytosolic Ca2+ handling and membrane potential regulation is a key causative factor. This study, for the first time, investigated the relationship between AP morphology and Ca2+ transient alternans in atria and compared the results with findings from ventricular tissue. In voltage-clamp experiments, we showed that Ca2+ transient alternans can be induced irrespective of the presence or absence of action potential duration alternans, however, suppression of Ca2+ release from sarcoplasmic reticulum abolished action potential duration alternans. Our work reveals new insights into mechanisms and regulation of alternans in atrial cells and demonstrates that atrial and ventricular myocytes share many similarities in the underlying mechanisms, but also exhibit some important differences. Nonetheless, common to both tissues is the key finding that the occurrence of electrophysiological alternans depends on beat-to-beat alternation of AP duration and morphology secondary to disturbances in Ca2+ signaling.
The Mechanisms of Calcium Cycling and Action Potential Dynamics in Cardiac Alternans
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Circ Res. 2015;116:846-856; originally published online December 22, 2014;
doi: 10.1161/CIRCRESAHA.116.305404
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/116/5/846

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/12/22/CIRCRESAHA.116.305404.DC1

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Supplemental Material

Detailed Methods

Myocyte isolation. Myocytes from the left ventricle and both atria were isolated from male New Zealand White rabbits (37 animals, ~2.5 kg, Harlan Laboratories, Indianapolis, IN, USA). Rabbits were anaesthetized with IV injection of sodium pentobarbital (50 mg/kg) and heparin (1000 UI/kg). Hearts were excised, mounted on a Langendorff apparatus and retrogradely perfused via the aorta. After an initial 5 min washing step with oxygenated Ca^{2+}-free Tyrode solution (in mmol/L: 140 NaCl, 4 KCl, 10 D-Glucose, 5 Hepes, 1 MgCl_2, 10 BDM, 1000 UI/l Heparin; pH 7.4 with NaOH), the heart was perfused with minimal essential medium Eagle (MEM) solution containing 20 µmol/L Ca^{2+} and 22.5 µg/mL Liberase TH (Roche Diagnostic Corporation, Indianapolis, IN, USA) for 20 min at 37˚ C. The left ventricle and both atria were dissected from the heart and minced, filtered and washed in a MEM solution containing 50 µmol/L Ca^{2+} and 10 mg/mL bovine serum albumin. Isolated cells were washed and kept in MEM solution with 50 µM Ca^{2+} at room temperature (22-24˚C) and were used within 1-8 h after isolation. All procedures and protocols involving animals conform with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee.

Electrophysiological measurements: Electrophysiological signals were recorded from single cardiac myocytes in the whole-cell ruptured patch clamp configuration using an Axopatch 200A patch-clamp amplifier, the Axon Digidata 1440A interface and pCLAMP 10.2 software (Molecular Devices, Sunnyvale, CA). Current and AP recordings were low-pass filtered at 5 kHz and digitized at 10 kHz.

The standard external Tyrode solution was composed of (in mmol/L): 140 NaCl, 4 KCl, 2 CaCl_2, 1 MgCl_2, 10 Hepes, 10 D-glucose; pH 7.4 with NaOH. All chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. For AP measurements and AP voltage clamp experiments patch pipettes (2–3 MΩ) were pulled from borosilicate glass capillaries (WPI, Sarasota, FL, USA) with a horizontal puller P-97 (Sutter Instruments, Novato, CA, USA) and filled with internal solution, containing (in mmol/L): 130 K⁺ glutamate, 10 KCl, 10 NaCl, 0.33 MgCl_2, 4 MgATP, and 10 Hepes with pH adjusted to 7.2 with KOH. For simultaneous [Ca^{2+}] measurements 75 µmol/L of Fluo-4 pentapotassium salt or 75 µmol/L Indo-1 pentapotassium salt (both from Molecular Probes/Life Technologies, Grand Island, NY) was added to the internal solution. Internal solutions were filtered through 0.22-µm pore filters. All experiments were performed at room temperature (22-24˚C).

For AP measurements the whole-cell 'fast' current clamp mode of the Axopatch 200A was used and AP were evoked by 5 ms stimulation pulses of ~1.5-2 times higher
magnitude than AP activation threshold. Cell membrane potential ($V_m$) measurements were corrected for a junction potential of -10 mV.

For AP-clamp experiments voltage commands in form of atrial or ventricular AP$_{CaT\_Small}$ and AP$_{CaT\_Large}$ were derived as the average of a typical AP$_{CaT\_Small}$ and AP$_{CaT\_Large}$ recorded from three individual cells at a stimulation rate of 1.3 Hz and exhibiting CaT alternans with CaT$_{Small}$/CaT$_{Large}$ ratio of ~0.4 (or AR=~0.6 as defined below). This resulted in two AP prototypes for each cell type that were used as command voltages, where AP$_{CaT\_Large}$ refers to the AP morphology recorded in connection with a large alternans Ca$^{2+}$ transient, whereas AP$_{CaT\_Small}$ was recorded during a small alternans Ca$^{2+}$ transient (cf. Fig. 1). Stimulation frequency was modified by proportionally changing AP duration and diastolic interval at rates >1.3 Hz, or by prolonging diastolic intervals between stimulations at frequencies <1.3 Hz. All AP-clamp experiments were conducted in standard Tyrode solution.

For I$_{LCC}$ recordings all K$^+$ ions in intracellular and extracellular solutions were replaced with Cs$^+$ to eliminate K$^+$ currents. External solution was composed of (in mmol/L): 140 NaCl, 4 CsCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 Heps, 10 D-glucose; pH 7.4 with NaOH. Pipettes were filled with internal solution, containing (in mmol/L): 130 Cs glutamate, 10 CsCl, 10 NaCl, 0.33 MgCl$_2$, 4 MgATP, and 10 Heps with pH adjusted to 7.2 with CsOH. For I$_{LCC}$ measurements cardiac myocytes were voltage clamped and held at -50 mV. I$_{LCC}$ was triggered by depolarization steps to 0 mV lasting 100 ms. Peak I$_{LCC}$ was measured as the difference between the inward peak current and the current at the end of the 100 ms depolarization pulse. For I$_{LCC}$ I-V relationship myocytes were depolarized from a holding potential of -50 mV to test potentials between -50 and +50 mV with increments of 5 or 10 mV.

**Cytosolic [Ca$^{2+}$]$_i$ measurements** Simultaneously with electrophysiological recordings cytosolic [Ca$^{2+}$]$_i$ levels were monitored. For [Ca$^{2+}$]$_i$ measurements cells were loaded with fluorescent probes Fluo-4 pentapotassium salt or Indo-1 pentapotassium salt via the patch pipette. Fluo-4 fluorescence was excited with the 488 nm line of an argon ion laser and [Ca$^{2+}$]-dependent Fluo-4 signals were collected at 515 nm using a photomultiplier tube. Background-subtracted fluorescence emission signals (F) were normalized to resting fluorescence ($F_0$) recorded under steady-state conditions at the beginning of experiment, and changes of [Ca$^{2+}$]$_i$ are presented as changes of $F/F_0$. Indo-1 fluorescence was excited at 357 nm (Xe arc lamp) and emitted cellular fluorescence was recorded simultaneously at 410 nm ($F_{410}$) and 485 nm ($F_{485}$) with photomultiplier tubes. $F_{410}$ and $F_{485}$ signals were background subtracted and changes of [Ca$^{2+}$]$_i$ were expressed as changes in the ratio $R=F_{410}/F_{485}$. Measurements, data recording and digitization were achieved using the Axon Digidata 1440A interface and pCLAMP 10.2 software.
CaT alternans was induced by incrementally increasing the pacing frequency (frequencies used: 0.5, 0.8, 1.0, 1.3, 1.6, 1.8, 2.0 Hz) until stable alternans was observed. The average frequencies at which stable CaT alternans was observed in current- and voltage-clamp experiments in atrial and ventricular myocytes are summarized in Supplemental Figure I. The degree of CaT alternans was quantified as the alternans ratio (AR). AR=1-CaT_{Small}/CaT_{Large}, where CaT_{Small} and CaT_{Large} are the small- and large-amplitude CaTs from a pair of alternating CaTs. CaTs were considered alternating when the beat-to-beat variation in CaT amplitude exceeded 10% (AR of >0.1)\(^1\). The amplitude of a CaT was measured as the difference in R or F/F\(0\) measured immediately before the stimulation pulse and the peak of the CaT.

**Data analysis and presentation.** Results are presented as individual observations or as means ± SEM. Statistical significance was evaluated using Student’s t-test. Unless stated otherwise, n represents the number of individual cells and differences were considered significant at p<0.05.

**References:**

Supplemental Fig. I. Alternans induction threshold for different current- and voltage-clamp protocols.

Alternans induction threshold was determined as the mean of the lowest pacing frequencies at which CaT alternans were observed with different pacing modes used. There were no significant differences between thresholds in current-clamp, same-shape AP-clamp or alternating AP-clamp experiments in both atrial and ventricular cells. However, a tendency to higher pacing rates to elicit CaT alternans was observed in voltage-clamp experiments using step-like depolarizations (I_{LCC} protocol). Also, ventricular cells tended to exhibit lower alternans induction thresholds than atrial myocytes.
Supplemental Fig. II. Application of ryanodine suppresses beat-to-beat alternation in APD.
Summary data for APD30, APD50 and APD90 before (control) and after application of 10 µmol/L ryanodine in atrial myocytes (n=4).