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Role of Calcium Cycling Versus Restitution in the Mechanism of Repolarization Alternans

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Abstract—T-wave alternans, a powerful marker of arrhythmic events, results from alternation in action potential duration (APD). The underlying cellular mechanism of APD alternans is unknown but has been attributed to either intracellular calcium (Ca\(^{2+}\)) cycling or membrane ionic currents, manifested by a steep slope of cellular APD restitution. To address these mechanisms, high-resolution optical mapping techniques were used to measure action potentials and Ca\(^{2+}\) transients simultaneously from hundreds of epicardial sites in the guinea pig model of pacing-induced T-wave alternans (n=7). The pacing rates (ie, alternans threshold) at which T-wave (369±11 bpm), APD (369±21 bpm), and Ca\(^{2+}\) (371±29 bpm) alternans first appeared were comparable. Importantly, the site of origin of APD alternans and Ca\(^{2+}\) alternans consistently occurred together near the base of the left ventricle, not where APD restitution was steepest. In addition, APD and Ca\(^{2+}\) alternans were remarkably similar both spatially and temporally during discordant alternans. In conclusion, the mechanism underlying T-wave alternans in the intact heart is more closely associated with intracellular Ca\(^{2+}\) cycling rather than APD restitution. (Circ Res. 2004;94:1083-1090.)

Key Words: electrophysiology ■ T-wave alternans ■ repolarization ■ Ca\(^{2+}\) cycling ■ optical mapping

Although we\(^{1,2}\) and others\(^{3-7}\) have shown that T-wave alternans in most circumstances results from alternation of cardiac repolarization at the cellular level, the mechanisms responsible for cellular alternans are not well understood. One hypothesis states that repolarization alternans arises when the heart rate exceeds the capacity of myocytes to cycle intracellular Ca\(^{2+}\)\(^{4-6,8-11}\). To maintain homeostasis, the amount of Ca\(^{2+}\) released from the sarcoplasmic reticulum (SR) to initiate contraction must be equal to the amount of Ca\(^{2+}\) reclaimed from the cytosol by SR Ca\(^{2+}\)-ATPase (SERCA). When one or more of the Ca\(^{2+}\) cycling proteins are impaired, the amount of released Ca\(^{2+}\) can only be fully reclaimed on an alternating heart basis, giving rise to alternation of free cytosolic Ca\(^{2+}\) levels. This, in turn, can cause alternans of membrane voltage (ie, repolarization) via several electrogenic Ca\(^{2+}\)-sensitive sarcolemmal currents. Reduced SERCA expression\(^{12}\) or interventions that decrease the function of SR Ca\(^{2+}\) release channels (Ryanodine receptors)\(^9\) have been shown to promote both intracellular Ca\(^{2+}\) alternans and action potential duration (APD) alternans (APD-ALT). Most studies, however, have focused on the mechanisms responsible for APD and intracellular Ca\(^{2+}\) alternans in single cell\(^{6,8,9,11}\) and tissue samples.\(^{3,5,12}\) The relationship between the emergence of alternans at the cellular level, ECG T-wave alternans, and arrhythmogenesis is not well understood.

A second hypothesis states that APD-ALT occurs when the slope of the APD restitution curve is >1, as determined by the kinetics of membrane currents.\(^{7,13-15}\) The slope of the APD restitution curve seems to play a critical role in APD-ALT and wavebreak.\(^{13,15-17}\) Several experimental\(^{16,17}\) and theoretical studies\(^{13,18}\) have shown that a slope >1 promotes alternans, whereas a slope <1 prevents it. Assuming this hypothesis is true, the threshold at which alternans first appears (ie, theoretical alternans threshold) can be calculated from the APD and diastolic interval (DI) when the restitution curve slope equals 1.

To date, no study has directly compared the relevance of cellular restitution and intracellular Ca\(^{2+}\) handling with the mechanism of repolarization alternans at the level of the whole heart. The guinea pig model of T-wave alternans\(^{1,2}\) includes a ventricular gradient of APD restitution that follows a base-to-apex pattern similar to the APD gradient, where longer APD, steeper restitution kinetics, and lower theoretical alternans threshold occur toward the base of the right ventricle (RV).\(^{19,20}\) Therefore, this model provides an opportunity to determine the relationship, if any, between the development of repolarization alternans and cellular restitution properties. Moreover, we used a novel method for simultaneously recording action potentials and Ca\(^{2+}\) transients\(^{21}\) to investigate the role of intracellular Ca\(^{2+}\) and APD restitution in the mechanism of repolarization alternans.
Materials and Methods

Experimental Preparation
Experiments were carried out in accordance with the United States Public Health Service guidelines for the care and use of laboratory animals. Adult guinea pig breeders (n=7, 800 to 1000 g) were anesthetized with pentobarbital sodium (30 mg/kg IP), and their hearts were rapidly excised and perfused as Langendorff preparations with oxygenated (95% O2 and 5% CO2) Tyrode solution containing (in mmol/L) NaCl 121.7, NaHCO3 20.0, MgSO4 1.18, KCl 4.81, dextrose 5.0, and CaCl2 1.25 (pH 7.40, 32°C).21 Hearts were first stained by direct coronary perfusion for ~10 minutes with the voltage-sensitive indicator di-4-ANEPPS (Molecular Probes) at a final concentration of 15 μmol/L and then for 45 minutes with the Ca2+-sensitive indicator indo 1-AM (Molecular Probes) at a final concentration of 5 μmol/L.21 In all experiments, a low concentration of 2.3-butanedione monoxime (5 mmol/L) was used to reduce motion artifact without altering substantially intracellular Ca2+ handling22 or cellular electrophysiology.23 The mapping field was carefully positioned over the left descending coronary artery.

Multisite Dual-Voltage Calcium Imaging
To selectively distinguish membrane voltage (Vm) from intracellular Ca2+ signals, it was essential to use dyes that avoid overlap in emission spectra. Therefore, we developed a system for simultaneous intracellular Ca2+ and Vm imaging. Excitation light for di-4-ANEPPS (515±5 nm) was obtained from a 200-W quartz tungsten halogen light source, and excitation light for indo-1 (365±25 nm) was obtained from a 200-W mercury arc lamp light source. Both were directed to the same position on the heart with separate liquid light guides.21 A dichroic mirror placed between tandem lens passed light of longer wavelengths to an emission filter (>695 nm) and photodiode array and reflected light of shorter wavelengths to a second emission filter (485±5 nm) and photodiode array.21 Both photodiode arrays were carefully aligned with an accuracy of 35 μm to assure recordings from similar locations.21 Signals recorded from each array and ECG signals were multiplexed and digitized with 12-bit precision at a sampling rate of 1 kHz/channel. For the present study, an optical magnification of ×1.24 resulted in a total mapping field of 14×14 mm, with 0.9 mm spatial resolution between sites.

Stimulation Protocol
Bipolar stimulation was performed at twice diastolic threshold using two Teflon-coated silver electrodes separated by 1 mm. Electrodes were carefully inserted into the myocardium near the apex of the left ventricle (LV) to reproduce as close as possible normal propagation (ie, from endocardium to epicardium and from apex to base). Alternans was induced by periods (30 seconds) of rapid pacing from the apex of the LV separated by resting periods of 30 seconds, during which preparations were allowed to recover at a pacing cycle length of 400 ms. Simultaneous Ca2+ and Vm signals were recorded for 10 seconds at the end of rapid pacing, which started at a pacing cycle length of 300 ms (200 bpm) and was decreased by 10 ms until 1/1 capture was lost or until ventricular fibrillation ensued. All measurements were made during steady-state alternans. In two of the seven experiments, this protocol was repeated while pacing from a different location (base of the LV).

Restitution Protocols
In a subset of experiments, standard and dynamic APD restitution were measured simultaneously from each recording site by introducing a single premature stimulus (S2) after a 40-beat drive train (S1) at the basic cycle length of 400 ms (ie, standard restitution)19 and by plotting APD as a function of DI measured during periods of rapid pacing used to promote alternans (ie, dynamic restitution).7,18,23

Data Analysis
Activation time and repolarization time were measured as described previously.1,2 Ca2+ alternans (CaF-ALT) was defined as the difference in the net amplitude of the large and small Ca2+ transient, expressed as a percent of the average net amplitude of two consecutive beats. APD-ALT and CaF-ALT were considered to be present when absolute differences in consecutive beats were ≥4 ms and >10%, respectively. These threshold values gave us a reliable measure of alternans spatially and temporally across all experiments. An epicardial region was considered alternating when at least 10 neighboring sites fulfilled criteria for APD-ALT or CaF-ALT. Concordant alternans was defined as one or more regions in phase, fulfilling criteria for APD-ALT or CaF-ALT, and spatially discordant alternans as two or more regions but in opposite phase, ie, one region showed a long-short APD or a large-small CaF amplitude pattern (positive difference) whereas another region showed a short-long APD or a small-large CaF amplitude pattern (negative difference).

APD restitution was fit to a single exponential to compare restitution kinetics characteristics between recording sites. The following parameters of APD restitution were calculated at each recording site: (1) APD0, the baseline APD; (2) τ, the time constant of a single exponential fit; (3) the DI and APD values, where slope equals 1 on the restitution curve13,16; and (4) the theoretical alternans pacing rate threshold, defined as [1000(APD+ΔI)/60]×60, where APD and DI values are taken from where slope equals 1 on the restitution curve13,16.

Statistics
Nonparametric tests were used to assess differences between paired and unpaired samples, respectively. P<0.05 was considered significant. Linear regression analysis was used to evaluate possible association between variables.

Results
Spatial and Temporal Relationships Between APD and Intracellular Ca2+ Alternans
Figure 1A shows representative examples of the ECG, action potential, and CaF transients recorded simultaneously at three different pacing rates. During baseline pacing (150 bpm), the ECG, APD, and CaF amplitude were stable over time and no alternans was observed. At a pacing rate of 375 bpm, subtle beat-to-beat alternans in APD, CaF amplitude, and T-wave amplitude was observed. An additional increase in pacing rate (461 bpm) resulted in pronounced alternans of APD, CaF amplitude, and T-wave amplitude was observed. An additional increase in pacing rate (461 bpm) resulted in pronounced alternans of APD, CaF amplitude, and T-wave alternans. In all experiments, long APDs were always associated with CaF transient of large amplitude and short APD with CaF transient of small amplitude (ie, discordant electromechanical alternans). Figure 1B displays the averaged magnitude of APD-ALT and CaF-ALT across the entire mapping field as a function of pacing rate for one representative experiment. By gradually increasing the pacing rate, an exponential increase in both APD-ALT and CaF-ALT occurred simultaneously at 340 bpm. In this example, the threshold pacing rate for inducing APD-ALT and CaF-ALT in all experiments was similar (Table), suggesting that the development of beat-to-beat APD-ALT closely coincides with the development of CaF-ALT.

For each experiment, we determined the spatial relationship between APD-ALT and CaF-ALT measured simultaneously from multiple sites across the anterior epicardial surface. In a representative example, at a pacing rate of 375 bpm (Figure 2, top), both APD-ALT and CaF-ALT were concordant in space and originated at the base of the LV, away from the apical stimulation site. In separate experiments, the site of alternans onset also occurred at the LV base,
even when pacing from the base of the LV. At a faster pacing rate, APD-ALT and CaF-ALT increased in magnitude and spread toward regions that were not previously alternating (ie, RV base and apex). In these regions, the cycle length at which alternans occurred was shorter (10 to 20 ms) compared with the site of alternans onset (LV base). Importantly, as pacing rate was increased (461 bpm, Figure 2, bottom), both APD-ALT and CaF-ALT became discordant in space, where neighboring regions alternated in opposite phase. The entire mapping field displayed beat-to-beat alternans in APD and CaF amplitude with bands of zero alternans separating regions in opposite phase (ie, node).

Table 1 shows similar pacing rate thresholds between spatially discordant APD-ALT and CaF-ALT in all experiments ($P=NS$). In a subset of experiments, similar results were observed for a pacing site located at the LV base. In summary, these data show that beat-to-beat APD and CaF amplitude were closely associated spatially both during concordant and discordant alternans and that the site of alternans onset was independent of pacing location.

**Site of Repolarization Alternans Onset**

Interestingly, the sites showing the first signs of APD-ALT or CaF-ALT were not randomly distributed across the mapping field but consistently occurred toward the base of the LV, independent of pacing site. We defined the region where alternans first appeared as the alternans-prone site, in contrast to regions where alternans occurred at faster pacing rates (ie, alternans-resistant site). Figure 3 shows representative examples of the ECG (top), action potentials, and APD time series from the alternans-prone site (middle) and an alternans-resistant site (bottom). At a pacing rate of 375 bpm, the alternans-
prone site displayed a subtle but visible beat-to-beat alternation in DI and APD. The time series below the action potential traces undoubtedly showed alternation in beat-to-beat APD of 10 ms. In contrast, the alternans-resistant site displayed no visible alternans, further illustrated by the lack of beat-to-beat APD-ALT in the time series shown below the traces. Note as well the subtle alternation in the amplitude of the ECG T wave also evident in the corresponding time series.

**Role of APD Restitution in Repolarization Alternans**

To investigate the mechanism of alternans onset, standard and dynamic restitution curves were compared between the alternans-prone site and an alternans-resistant site near the RV base in a subset of experiments. Figures 4A and 4B show standard restitution curves from two representative examples. In both experiments, the alternans-prone site is characterized by shorter baseline APD value ($APD_b$), shallower kinetics (ie, longer restitution time course ($\tau$)), shorter DI at slope of 1, and higher theoretical alternans heart rate threshold (HR$_{alt}$) compared with the alternans-resistant site ($APD_d$, $HR_{alt}$). Similarly, the DI at which alternans thresholded by a shorter baseline APD value ($APD_d$), shallower kinetics (ie, longer $\tau$), shorter DI at slope of 1, and higher theoretical alternans heart rate threshold (HR$_{alt}$) compared with the alternans-resistant site ($HR_{alt}$).
occurred at the LV base (34±3 ms) was longer by 10 ms than the predicted value based on the standard restitution curve (22 to 24 ms, Figure 4). These data suggest a lack of causal relationship between APD-ALT and kinetics of AP restitution as assessed by the standard restitution protocol.

To further investigate the mechanism of alternans, we compared the spatial pattern of alternans onset with baseline APD gradients. In general, APD gradients were oriented from the RV base to the LV free wall, orthogonal to the LAD artery, as shown in Figure 5A. Interestingly, alternans did not start where APD was longest, ie, at the base of the RV, nor where APD was shortest, ie, toward the LV free wall. In fact, alternans consistently started at the base of the LV, where APD values tended to be intermediate (Figure 5A, hatched area). Figure 5B shows that for all experiments (n=7), mean baseline APD at the alternans-prone site was significantly smaller than at the alternans-resistant site at the RV base (211±8 versus 219±8 ms, respectively; P<0.01). Similarly, we compared the pattern of alternans onset with the time course of standard APD restitution, as described by τ in the same representative experiment shown in Figure 5A. As expected, τ followed the APD gradient, with steeper kinetics (ie, smaller τ) toward the RV and shallower kinetics (ie, longer τ) toward the LV free wall. Interestingly, the site of earliest alternans was not located where APD restitution kinetics were steepest (ie, RV) but occurred where standard restitution kinetics were shallow (ie, toward the base of the LV). Figure 5D shows a plot of τ from the standard restitution curve as a function of APD-ALT magnitude in the same representative example in Figures 5A and 5C. APD-ALT magnitude was measured at a pacing rate (400 bpm) at which the anterior epicardial surface showed alternans that was only concordant in space. Assuming that standard APD restitution kinetics govern alternans in the intact heart, one would expect that τ would be smallest (ie, steep kinetics) where APD-ALT is largest and that τ would be largest (ie, shallow kinetics) where APD-ALT is smallest. As indicated by the low correlation coefficient (R²=0.06), no relationship could be established between the time course of standard APD restitution and APD-ALT magnitude.

Figure 6A shows dynamic restitution curves from the alternans-prone site (LV base) and alternans-resistant site (RV base). Interestingly, both sites showed similar kinetics, with a time constant (τ) of ≈100 ms and a DI of 30 ms, reaffirming that susceptibility of a cell to alternans is not determined by its restitution properties. Figure 6B shows that the measured alternans threshold (DI, 34±3 ms) is significantly smaller than the predicted alternans threshold based on the dynamic restitution curves for each experiment (DI, 54±8 ms). Taken together, these data suggest that in the intact heart, there is no causal relationship between APD-ALT and APD restitution kinetics in its broad definition (ie, standard and dynamic).

**Discussion**

The present study shows that the mechanisms underlying T-wave alternans in the intact heart are more closely associated with intracellular Ca²⁺ cycling rather than APD restitution (ie, standard and dynamic). We also found that alternans of both APD and intracellular Ca²⁺ consistently originated at the base of the LV and were unrelated to APD restitution kinetics in the intact heart.
analyzed the relationship between alternans and dynamic restitution, which takes into account pacing history (ie, memory). Interestingly, measured DI at which alternans was first observed was significantly shorter than the predicted DI at a slope of 1, measured from dynamic restitution curves reaffirming the poor relationship between repolarization alternans and APD restitution.

Our findings share some similarities with previous studies showing a poor relationship between restitution kinetics and APD-ALT.5,7,24 Banville et al7 observed stable alternans in the intact heart at rates where the slope of the APD restitution curve <1. In addition, Saitoh et al3 reported that transient APD-ALT in muscle fibers was dependent on intracellular Ca2+ cycling rather than on the recovery of membrane currents, as measured by APD restitution. Our study extends their findings to stable alternans in the intact heart, which, unlike transient alternans, is proven to increase risk of arrhythmic events.1,2,25

Assuming that APD restitution slope >1 promotes alternans whereas slope <1 prevents it,3,16–18 the theoretical alternans threshold can be calculated from the APD and diastolic interval values where the slope equals 1 (see the Materials and Methods section for details). However, it is well established clinically3,26 and experimentally27 that increasing the baseline pacing rate at which restitution is measured moves the APD restitution downward and leftward. This is, in turn, produces multiple theoretical alternans thresholds, which may preclude the use of restitution (a measure accessible during clinical electrophysiology study3,26) as a simple means to clinically determine alternans threshold. Taken together, these data suggest a lack of causal relationship between APD-ALT and APD restitution kinetics in the intact heart. However, cardiac memory or electronic effects may account for this discrepancy.5,24 Interestingly, we have found that calcium cycling also seems to play a role in short-term cardiac memory, which, in turn, can modulate the alternans heart rate threshold.28 In addition, using theoretical models, it has been shown that short-term memory may be caused by factors other than calcium cycling, such as K+ current deactivation.29

Finally, APD restitution and intracellular Ca2+ cycling are not mutually exclusive causes of APD alternans, because one may affect the other. However, in different experimental conditions that affect APD restitution and intracellular Ca2+ cycling differentially (eg, sympathetic tone, different [Ca2+]i, and temperature), one or the other mechanism may dominate. It is also important to emphasize that APD restitution can provide a useful approach for understanding alternans but additional investigation of calcium cycling proteins must be pursued to ascertain the underlying cellular mechanisms.

Intracellular Ca2+ Cycling as a Mechanism of Repolarization Alternans

Another original finding in our study is that both APD and intracellular Ca2+ alternans consistently originated at the base of the LV, independent of pacing site. At the alternans threshold, APD and intracellular Ca2+ were both concordant in space and confined to a relatively small region near the LV base. Spatial heterogeneity of intrinsic cellular function may
play a role in the preferential onset of alternans toward the base of the LV. Electrophysiological properties\textsuperscript{19,20} and contractility\textsuperscript{30} are known to follow a base-to-apex gradient across the heart. In particular, the base of the heart displays a weaker contraction and slower relaxation\textsuperscript{30} as well as Ca\textsuperscript{2+} transients of longer duration\textsuperscript{20,31} and smaller amplitude\textsuperscript{31} compared with the apex. Such heterogeneity of Ca\textsuperscript{2+} handling may provide insights into the cellular mechanisms of alternans. For example, a smaller amplitude of Ca\textsuperscript{2+} release near the base of the heart is indicative of weaker Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release. Both mechanical and repolarization alternans have been associated with alternans of Ca\textsuperscript{2+} release by the SR on a beat-to-beat basis.\textsuperscript{4,5,8,12,15} L-type Ca\textsuperscript{2+} blockers, which affect membrane current and Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, decreased but did not prevent mechanical alternans.\textsuperscript{10} In contrast, pharmacological compounds preventing the release of Ca\textsuperscript{2+} from the SR (eg, Ryanodine and caffeine) abolished both APD and intracellular Ca\textsuperscript{2+} alternans. Recently, reduced SERCA expression\textsuperscript{12} or interventions that decrease Ryanodine receptor function, such as reducing the open probability or availability of glycolytic ATP,\textsuperscript{9} have been shown to promote both intracellular Ca\textsuperscript{2+} and APD alternans. Most studies, however, have focused on the mechanisms responsible for APD and intracellular Ca\textsuperscript{2+} alternans in single cell\textsuperscript{8,9,11} and tissue samples.\textsuperscript{5,5,12} Because our measurements were made from the anterior epicardial surface of the LV and RV, the earliest site of alternans onset could have occurred elsewhere. However, APD and Ca\textsuperscript{2+} alternans thresholds were similar to the ECG T-wave alternans threshold (Table), suggesting that the alternans observed first at the LV base reflected the earliest site of onset. In conclusion, our data suggest that Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release may also play a role in the onset of alternans in the intact heart and, moreover, that the base of the LV has unique Ca\textsuperscript{2+}-handling properties rendering this region prone to alternate first.

We always observed concordant electromechanical alternans during spatially concordant and discordant alternans. This finding is similar to experiments performed by Choi et al\textsuperscript{20} in the intact guinea pig heart and by Rubenstein et al\textsuperscript{8} in single cells, both of which were performed at near-physiological temperatures. Orchard et al\textsuperscript{8} have shown that the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange current is involved in promoting APD-ALT. However, discordant electromechanical alternans has been reported when experiments were run at room temperatures.\textsuperscript{5,32} Interestingly, the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange current is reduced at room temperature more than other ionic membrane currents.\textsuperscript{6} Thus, it is possible that other Ca\textsuperscript{2+}-sensitive currents (I_{Na\textsuperscript{+}} and I_{K\textsuperscript{+}}) dominate at low temperature, resulting in electromechanical discordant alternans. We occasionally noticed a peculiar pattern at very rapid pacing rates where the peak of the short AP upstroke appeared to raise slightly above the longer AP upstroke (Figure 3). The mechanisms of this is not clear but may be related to supernormal excitability, where the takeoff potential is slightly higher than resting potential but still lower than the potential at which sodium channels inactivate.\textsuperscript{33}

Our observation of a close spatial and temporal association between intracellular Ca\textsuperscript{2+} and APD during alternans is in good agreement with previous studies performed in nonischemic\textsuperscript{30} and ischemic preparations.\textsuperscript{34} It is important to mention, however, that the onset of alternans may be different during regional ischemia. The close temporal and spatial association between APD and intracellular Ca\textsuperscript{2+} reported in the present study suggests that Ca\textsuperscript{2+} cycling may also play a role in the pathogenesis of discordant alternans. When the pacing rate is increased, intracellular Ca\textsuperscript{2+} rises to a new steady-state level.\textsuperscript{12,20} Increasing intracellular Ca\textsuperscript{2+} has been shown to promote cell uncoupling independent of intracellular H\textsuperscript{+}.\textsuperscript{35} A decrease in coupling between regions or cells may also enhance spatial heterogeneity of electrophysiological properties\textsuperscript{3} and slow impulse propagation across the heart,\textsuperscript{36} each of which has been shown to lower alternans threshold\textsuperscript{2} and enhance discordant alternans.\textsuperscript{2,13,14,18} However, additional studies are required to elucidate the role played by intracellular Ca\textsuperscript{2+} in spatially discordant alternans.

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