Hysteresis Effect Implicates Calcium Cycling as a Mechanism of Repolarization Alternans

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Background—T-wave alternans is due to alternation of membrane repolarization at the cellular level and is a risk factor for sudden cardiac death. Recently, a hysteresis effect has been reported in patients whereby T-wave alternans, once induced by rapid heart rate, persists even when heart rate is subsequently slowed. We hypothesized that alternans hysteresis is an intrinsic property of cardiac myocytes, directly related to an underlying mechanism for repolarization alternans that involves intracellular calcium cycling.

Methods and Results—Stepwise pacing was used to induce alternans in Langendorff-perfused guinea pig hearts from which optical action potentials were recorded simultaneously at 256 ventricular sites with voltage-sensitive dyes and in whole-cell patch-clamped cardiac myocytes treated with or without BAPTA-AM (1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid tetrakis [acetoxymethyl ester]). Alternans hysteresis was observed in every isolated heart: threshold heart rate for alternans was 280±12 bpm, but during subsequent deceleration of pacing, alternans persisted to significantly slower heart rates (238±5 bpm, P<0.05). Optical mapping showed that this effect also applied to the threshold for spatially discordant alternans (313±2.2 bpm during acceleration versus 250±6.6 bpm during deceleration, P<0.05). Alternans hysteresis was also observed in isolated cardiac myocytes. Moreover, calcium chelation by BAPTA-AM raised the threshold for alternans and inhibited hysteresis in a dose-dependent manner with no effect on baseline action potential duration.

Conclusions—Alternans hysteresis is an intrinsic property of cardiac myocytes that can lead to persistence of arrhythmogenic discordant alternans even after heart rate is slowed. These results also support an important underlying role of calcium cycling in the mechanism of alternans. (Circulation. 2003;108:2704-2709.)

Key Words: electrophysiology ■ calcium ■ arrhythmia

T-wave alternans (TWA) has been closely associated with ventricular arrhythmias and sudden cardiac death in a wide variety of experimental and clinical conditions. Previously, we demonstrated that microvolt-level, visually undetectable TWA is common in patients at risk for sudden cardiac death and arises from alternations of repolarization that occur at the level of the single cell. These and other studies indicate that repolarization of cells in neighboring regions of myocardium can alternate asynchronously (ie, discordant alternans), forming spatial gradients of repolarization of sufficient magnitude to cause conduction block and reentrant arrhythmias.

Although TWA is a substrate for arrhythmogenesis, the mechanism whereby membrane potential alternates in response to an elevation of heart rate remains unclear. Although a variety of sarcolemmal currents can exhibit alternating-type activity, including the transient outward potassium current (Ito), the L-type calcium current (IKCa),10 and the inward rectifying potassium current (IK1),11 none have been implicated definitively in the mechanism of alternans. However, there are compelling data that suggest a primary role of intracellular calcium cycling. Alternans has been inhibited by (1) blockade of the ryanodine-sensitive Ca-release channel (Ryr) of the sarcoplasmic reticulum, (2) blockade of the L-type Ca channel with verapamil or nisoldipine, and (3) depletion of sarcoplasmic reticulum Ca stores with caffeine. Importantly, several investigators have observed alternans of contraction and/or intracellular Ca transients that occur in association with alternans of membrane potential.

A recent study suggesting that alternans exhibits hysteresis provides further support for a role of Ca in the mechanism of alternans. Narayan and Smith reported that TWA was of greater magnitude during decelerated pacing than for the same heart rate during accelerated pacing in patients undergoing electrophysiological testing. We hypothesized that...
alternans hysteresis is an intrinsic property of cardiac myocytes that is directly related to the mechanism for repolarization alternans and occurs when heart rate exceeds the ability of the cell to effectively cycle calcium.

Methods

Isolated Myocyte Experiments

Guinea pig ventricular myocytes were isolated by standard enzymatic dispersion techniques.21 Myocytes were resuspended in 10 mL of DMEM, stored at room temperature, and used within 12 hours. Cells were bathed in Tyrode’s solution (in mmol/L: NaCl 137, KCl 5.4, CaCl2 2, MgSO4 1, glucose 10, HEPES 10, pH 7.35 with NaOH), and action potentials (APs) were recorded by the nystatin-perforated patch whole-cell recording method (240 µg/mL nystatin in dimethylsulfoxide).22 Square pulse currents (5-ms duration, amplitude 1.5 to 2 × threshold) generated APs, and action potential duration (APD) was measured at 90% repolarization (APD90).

At each cycle length, alternans was measured as the difference in APD90 between 2 successive APs. Pacing rate was increased from baseline (1000 ms) by stepwise increments of 50 or 20 ms until the magnitude of alternans was >150 ms or cycle length was 200 ms, then decreased in reverse order of cycle length (n=12 cells). Threshold heart rate for alternans was the slowest heart rate that resulted in alternans ≥10 ms in either the forward (acceleration) or reverse (deceleration) direction (ALTf and ALTr, respectively). Alternans hysteresis was quantified as ALTf minus ALTr and measured in cells treated with or without 0.25, 0.38, or 0.5 µmol/L BAPTA-AM (1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid tetraakis [acetoxymethyl ester]; n=6 each group). All experiments were performed at room temperature.

Guinea Pig Whole-Heart Experiments

Hearts from 5 male retired-breeder guinea pigs were Langendorff perfused with oxygenated (95% O2, 5% CO2) Tyrode’s solution (in mmol/L: NaCl 130, NaHCO3 25.0, MgSO4 1.2, KCl 4.75, dextrose 5.0, CaCl2 1.25, pH 7.40, 26°C). The endocardial surface was eliminated by a cryoablation procedure described previously.26 Hearts were stained with 100 mL of the voltage-sensitive dye di-4-ANEPPS (20 µmol/L) by direct coronary perfusion for 10 minutes.

Optical APs were recorded with high voltage (0.5 mV) and temporal (1 ms) resolution from 256 sites across the anterior epicardial surface (see references26,27 for details). The tandem lens system has been described previously.26 An optical magnification of 1.2× was used, which corresponds to a total mapping area of 15×15 mm and 1.25-mm interpixel spatial resolution.

A previously validated experimental model of pacing-induced steady-state T-wave alternans6 was used, modified to include a deceleration phase (“reverse pacing”) for hysteresis measurements. The left ventricle was stimulated at a baseline heart rate of 500 ms (120 bpm) and increased stepwise at 1-minute intervals in increments of 50, 20, and then 10 ms until the stimulus artifact so impinged on the ventricular electrogram that further pacing would likely result in loss of capture or ventricular fibrillation (TWA was always visible on the ECG by this point). The pacing sequence was then reversed (ie, deceleration of pacing) following the same order of cycle length back down to baseline.

Alternans was determined at each heart rate after 1 minute of stimulation and measured by calculating the difference in APD (using previously described algorithms26,27) on 2 consecutive beats for each channel in the mapping field (ie, beat 1 minus beat 2). The absolute values of these numbers were averaged, and alternans was considered significant if ≥10 ms.6 ALTf and ALTr were defined as the lowest heart rate at which alternans was significant for pacing in the forward and reverse directions, respectively.

To explore whether alternans hysteresis affected discordant alternans, alternans at each ventricular site was mapped simultaneously across the ventricular surface by 2D contour maps. For this analysis, both the magnitude and phase of alternans were compared between all recording sites; hence, regions that alternated out of phase were assigned alternans values of opposite sign (ie, positive versus negative values). ALTf and ALTr for discordant alternans were the slowest heart rates at which there were at least 2 regions alternating with opposite phase in the forward and reverse directions, respectively.

Statistical Analysis

Statistical tests included 1-way ANOVA and Student-Newman-Keuls (BAPTA-AM experiments) or Student’s t test (paired, when relevant). Statistical significance was assessed at 5%. All data are mean±SEM, unless stated otherwise.

Results

Alternans Hysteresis in Intact Hearts

Figure 1A shows representative optical APs recorded from a single ventricular site during stepwise pacing in an intact guinea pig heart. During forward pacing (open arrows), alternans developed and increased in magnitude with increasing heart rate. During reverse pacing (solid arrows), alternans persisted as heart rate was decreased. Thus, the magnitude of alternans was greater in the reverse direction than in the forward direction at every heart rate tested (ie, hysteresis). Figure 1B shows alternans averaged from all recording sites during the same experiment. Asterisks indicate significantly greater alternans in the reverse than in the forward direction at each heart rate. Alternans threshold was higher in the
forward (ALT_F) than in the reverse (ALT_R) direction. Data for these experiments are summarized in the Table.

Alternans hysteresis was also observed with respect to discordant alternans. Figure 2 shows contour plots of alternans and phase in a representative experiment. During pacing in the forward direction (open arrows), significant alternans developed at 250 bpm, but all regions were alternating in phase. As rate was increased, alternans became discordant (ALT_F=300 bpm). When pacing was reversed (solid arrows), the 2 discordant regions of epicardium remained out of phase while the magnitude of alternans decreased with rate. In other words, there was no downward transition from discordant to concordant alternans during reverse pacing. This pattern was observed in all experiments. In Figure 2, ALT_F for discordant alternans was 230 bpm. Thus, discordant alternans persisted at rates much lower than those required to induce it. These data are summarized in the Table.

### Alternans Hysteresis in Isolated Myocytes

A representative example of APs recorded from isolated myocytes is shown in Figure 3. As in the intact-heart experiments (Figure 1), alternans appeared as heart rate was increased (open arrows) and persisted even when heart rate was subsequently decreased (solid arrows), ie, hysteresis.

We hypothesized that alternans occurs when heart rate exceeds the capacity of the myocyte to cycle Ca, and hence, alternans hysteresis may arise from Ca accumulation associated with sustained elevation of heart rate. Figure 4A shows APs from a representative experiment in a cell treated with 0.5 μmol/L BAPTA-AM (intracellular calcium chelator). These data are plotted in Figure 4B and compared with a representative control experiment. Two major effects were observed: (1) 0.5 μmol/L BAPTA-AM increased ALT_F compared with control, and (2) there was no significant difference between ALT_F and ALT_R in the BAPTA-AM experiment (ie, hysteresis was abolished).

The effects of BAPTA-AM were concentration dependent. Figure 5 shows that despite having no effect on baseline APD (top), ALT_F and ALT_R were significantly increased by 0.38 and 0.5 μmol/L BAPTA-AM compared with controls (mid-
Alternans hysteresis diminished progressively with increasing concentrations of BAPTA-AM and was significantly less than control at 0.5 μmol/L BAPTA-AM (bottom of Figure 5; n=6 cells per group).

The effect of 0.5 μmol/L BAPTA-AM on rate-dependent adaptation of APD was evaluated with APD90 values measured at cycle lengths of 1000 and 400 ms. Rate adaptation for APD (1000 ms: control=411±12 bpm, BAPTA=412±44 bpm; 400 ms: control=298±14 bpm, BAPTA=287±39 bpm) was not affected by BAPTA-AM, which indicates that the inhibition of alternans hysteresis by BAPTA-AM was attributable to reductions in cytoplasmic calcium load rather than changes in AP properties, including APD restitution.

**Discussion**

The present study shows that alternans exhibits a hysteresis effect whereby alternans persists at heart rates below those required to induce it. Furthermore, the results show hysteresis to be an intrinsic property of cardiac myocytes and suggest an important underlying role of calcium cycling in the mechanism of alternans.

**Cardiac Memory, Hysteresis, and Alternans Dynamics**

Cardiac memory, in the context of repolarization kinetics, may be defined as the dependence of APD on the "electrical history" of the tissue, rather than merely the characteristics of the preceding beat. Studies exploring the role of cardiac memory in APD alternans have generally focused on restitution curves and their rate dependence. The findings suggest that restitution alone, as determined by standard S1-S2 protocols, may be unable to fully account for the dynamics of alternans in ventricular myocytes or for more complex events, such as period doubling and chaotic dynamics, observed experimentally. However, when restitution is measured in such a manner as to consider the effect of cardiac memory (ie, "dynamic restitution"), more accurate predictions of alternating behavior can be made.

Furthermore, the slope of dynamic restitution curves is greater than S1-S2 restitution curves, and the restitution hypothesis requires a slope >1 for alternans to occur. Therefore, APD alternans may depend not just on the previous diastolic interval but also on a greater time context of electrical events (ie, cardiac memory).

The physiological consequence of restitution in the mechanism of alternans includes a hysteresis effect, whereby alternans is not solely dependent on heart rate, but rather the
direction of the heart rate change. The present study demonstrated that alternans hysteresis exists in intact hearts and is a property of individual cardiac myocytes that is related to intracellular calcium cycling.

**Hysteresis Effect for Discordant Alternans**

The present results showed that alternans hysteresis also applies to discordant alternans, a particularly arrhythmogenic form of alternans that provides a substrate for reentry. In fact, the hysteresis effect for discordant alternans was even greater than for concordant alternans. It has been shown that during forward pacing (acceleration), discordant alternans is preceded by concordant alternans, in which all regions of epicardium are alternating in phase. The present study shows that when pacing sequence is reversed (deceleration), discordant alternans does not necessarily develop from discordant alternans. Rather, regions of epicardium continue to alternate out of phase while the magnitude of alternans in each region decreases with rate.

These findings have important clinical implications, suggesting that the hysteresis effect may be strongest for the most arrhythmogenic form of alternans (discordant alternans). It has been shown that mild exercise can incite TWA in patients at risk for sudden cardiac death, with threshold heart rates in the range of 100 bpm compared with over 120 bpm in normal subjects. However, if TWA is related to the mechanism for sudden cardiac death, as suggested previously, it may not explain why many patients have episodes of sudden death that occur at heart rates below 100 bpm. The results of the present study provide a possible explanation for a number of factors that may be involved in the pathogenesis of TWA. The hysteresis effect for discordant alternans suggests that alternans hysteresis may also provide an important clue to the mechanism of repolarization alternans. Because intracellular calcium cycling can cause cytosolic Ca to alternate, our results support to an important role of intracellular Ca cycling in the mechanism of repolarization alternans. Despite having a significant effect on cellular alternans and hysteresis, BAPTA-AM did not affect APD or rate-dependent APD adaptation, which suggests that alternans hysteresis was caused by elevated cytosolic calcium rather than sarcolemmal currents.

Some limitations to the present study should be noted. Because intracellular calcium was not measured directly, one cannot be certain that BAPTA-AM diminished hysteresis by lowering cytosolic Ca when heart rate was elevated. However, because relatively low doses of BAPTA-AM were used, and because the observed effects were dose dependent, it is unlikely that the inhibitory effect of BAPTA-AM on alternans and hysteresis was due to nonspecific drug effects. Furthermore, the complex interaction between various aspects of intracellular calcium cycling, including sarcolemmal ion channels, makes it difficult to determine with any certainty the specific subcellular mechanisms responsible for alternans and alternans hysteresis from the present data. We also made no attempt to assess susceptibility to arrhythmias, so we cannot determine to what extent hysteresis or its attenuation by BAPTA would impact alternans-induced arrhythmogenesis. However, clinical evidence showing a strong link between alternans thresholds and relative risk of arrhythmic events does support such suggestions.

**Hysteresis Implicates Calcium in the Mechanism of Alternans**

Alternans hysteresis may also provide an important clue to the mechanism of cellular repolarization alternans. Because hysteresis manifests as a persistence of alternans, the mechanisms responsible for hysteresis are probably closely related to those that cause cells to alternate in the first place. We have hypothesized that alternans occurs when heart rate exceeds the capacity of the myocyte to cycle Ca. To maintain homeostasis, the amount of Ca entering the cell during each heart beat must be fully reclaimed from the cytoplasm. Therefore, impairment in the kinetics of any component of cellular Ca cycling can cause cytosolic Ca to alternate. Our data suggest that a persistent elevation of heart rate (during acceleration phase), by raising cytosolic Ca, caused repolarization alternans to persist even at lower heart rates (during deceleration phase).

It has been known for several decades that alternans of APD is associated with alternans of tension (ie, mechanical alternans), and alternans is inhibited or suppressed by verapamil, caffeine, BayK8644, nisoldipine, and ryanodine. Recently, it has been demonstrated in isolated rabbit and cat ventricular myocytes that Ca transient alternans can occur during rapid pacing in voltage-clamped cells in which there is no alternation of pulse duration. An elegant study by Hüser et al suggested that alternation of calcium-induced calcium release may be responsible for alternans. They found no alternation of L-type Ca current amplitude or sarcoplasmic reticulum Ca load; however, they were able to induce alternans by metabolic inhibition, which deprives the cell of ATP and affects glycolytic enzymes closely associated with the ryanodine release channel of the sarcoplasmic reticulum.

We used low concentrations of BAPTA-AM sufficient to inhibit but not completely prevent alternans to demonstrate that chelation of intracellular free Ca, which is expected to reduce the burden of Ca on the cycling mechanisms of the cell, raises alternans thresholds to higher heart rates and inhibits alternans hysteresis. These findings lend further support to an important role of intracellular Ca cycling in the mechanism of repolarization alternans. Despite having a significant effect on cellular alternans and hysteresis, BAPTA-AM did not affect APD or rate-dependent APD adaptation, which suggests that alternans hysteresis was caused by elevated cytosolic calcium rather than sarcolemmal currents.

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