Transmural Heterogeneity of Calcium Handling in Canine

Kenneth R. Laurita, Rodolphe Katra, Barbara Wible, Xiaoping Wan, Michael H. Koo

Abstract—Spatial heterogeneity of the action potential and its influence on arrhythmia vulnerability is known. However, heterogeneity of intracellular calcium handling and, in particular, its effect on the electrophysiological substrate is less clear. Using optical mapping techniques, calcium transients and action potentials were recorded simultaneously from ventricular sites across the transmural wall of the arterially perfused canine left ventricular wedge preparation during steady-state baseline pacing and rapid pacing. During baseline pacing, the decay of intracellular calcium to diastolic levels and calcium transient duration were slower (70%, \( P<0.005 \)) and longer (20%, \( P<0.005 \)), respectively, closer to the endocardial surface compared with the epicardial surface. Tissue samples isolated from the left ventricular wall demonstrate that sarcoplasmic reticulum \( Ca^{2+} \) ATPase (SERCA2a) expression was significantly less in the subendocardial and midmyocardial layers compared with the subepicardial layer. In contrast, no significant difference in the transmural expression of \( Na^{+}\)-\( Ca^{2+} \) exchanger was observed. During rapid pacing, calcium transient alternans and increased levels of diastolic intracellular calcium were significantly greater (\( P<0.01 \)) closer to the endocardium (101\% \( \pm \) 62\% and 41\% \( \pm \) 15\%, respectively) compared with the epicardium (12\% \( \pm \) 7\% and 12\% \( \pm \) 14\%, respectively). In conclusion, cells closer to the endocardium exhibit a slower decay of intracellular calcium compared with cells near the epicardium, which may be due in part to reduced expression of SERCA2a. As a possible consequence, calcium transient alternans and increased diastolic levels of intracellular calcium may occur preferentially closer to the endocardial surface. (Circ Res. 2003;92:668-675.)

Key Words: intracellular calcium ■ optical mapping ■ alternans ■ \( Na^{+}\)-\( Ca^{2+} \) exchanger ■ SERCA2a

Spatial heterogeneity of the action potential and its influence on arrhythmia vulnerability has been well described.\(^{1–3}\) However, heterogeneity of intracellular calcium handling and, in particular, its effect on arrhythmogenesis is less clear. Molecular studies suggest that a transmural heterogeneity of calcium regulatory proteins may exist. For example, reduced mRNA levels of sarcoplasmic reticulum (SR) \( Ca^{2+} \) ATPase (SERCA2a)\(^{4} \) and a trend toward less SERCA2a expression\(^{5} \) have been observed near the endocardium compared with the epicardium in normal hearts. At the level of the whole heart, Figueredo et al\(^{6} \) demonstrated transmural heterogeneities of the intracellular calcium transient that suggest intrinsic differences in calcium homeostasis from epicardium and endocardium. Taken together, these studies suggest that under normal conditions the recovery of intracellular calcium during diastole may be slower in cells near the endocardial surface of the heart (subendocardial) compared with cells near the epicardial surface (subepicardial).

Normally, the heart is not prone to arrhythmias and, thus, the electrophysiological consequence of transmural heterogeneity of calcium handling is not obvious. However, under certain conditions such heterogeneity could create regions across the transmural wall that are more susceptible to calcium-mediated arrhythmias. For example, repolarization alternans may be dependent on cycling of intracellular calcium\(^{7–11} \) and has been mechanistically linked to the initiation of ventricular arrhythmias.\(^{3,12} \) In addition, catecholamine-dependent arrhythmias\(^{13} \) might be triggered by delayed afterdepolarizations\(^{13} \) caused by intracellular calcium accumulation and spontaneous release of calcium from the SR. Therefore, it is possible that transmural heterogeneities of calcium recovery may, under abnormal conditions, form regions that are more susceptible to alternans and accumulation of intracellular calcium and provide a substrate for arrhythmias. We hypothesized that across the normal intact transmural wall there are intrinsic heterogeneities of intracellular calcium handling that are determined by the expression of calcium regulatory proteins. To test this hypothesis, optical mapping and protein expression studies were used to measure calcium transients, action potentials, and calcium regulatory protein expression across the canine transmural wall under control conditions.

Materials and Methods

Experimental Preparation

Experiments were carried out in accordance with Public Health Service guidelines for the care and use of laboratory animals.
Mongrel dogs (20 to 25 kg) were anticoagulated with heparin (2 cm$^3$) and anesthetized with sodium pentobarbital (30 mg/kg intravenously), and the hearts were removed by a left lateral thoracotomy and placed in cold (4°C) cardioplegia solution. Transmural wedges of cardiac tissue (in cm, 2.0 [height]×1.0 [width]×0.5 [depth]) surrounding and parallel to branches of the left anterior descending coronary artery were dissected from the left ventricular free wall (n=11). Approximately 50% of all preparations were taken near the base of the left ventricle. Free-running Purkinje fibers were removed from the endocardial surface. The coronary artery that perfuses each wedge was cannulated and perfused with oxygenated (95% O$_2$/5% CO$_2$) Tyrode solution containing (in mmol/L) NaCl 130, NaHCO$_3$ 20.0, NaH$_2$PO$_4$ 0.9, MgSO$_4$ 0.5, KCl 4.00, dextrose 5.5, and CaCl$_2$ 1.80 (pH 7.40). The wedge was perfused with the voltage-sensitive dye di-4-ANEPPS (15 μmol/L), and then was loaded for 30 minutes with a calcium-binding fluorescent dye, Indo-1 acetoxymethyl ester (5 μmol/L) dissolved in DMSO, followed by a 15- to 30-minute washout period. After perfusion with the fluorescent dyes, the wedge was perfused with normal Tyrode solution and transferred to a controlled flow pump. We have previously found these preparations to be stable for >2 hours. A unique advantage of this preparation is that multiple cell types can be investigated simultaneously in the same preparation. In 4 of 11 experiments, 2,3-butanedione monoxime (BDM, 5 mmol/L) was used to ensure that motion artifact, if any, was eliminated. In 4 of 11 experiments, 2,3-butanedione monoxime (BDM, 5 mmol/L) was used to ensure that motion artifact, if any, was eliminated.

Optical Mapping System

We have previously developed a dual calcium-voltage optical mapping system that is capable of measuring high-fidelity calcium transients and action potentials with high spatial and temporal resolutions simultaneously at multiple recording sites from the intact heart. Briefly, excitation light obtained from a 180-W quartz tungsten halogen lamp and a 250-W mercury arc lamp light source (Oriel Corp) was directed to the heart using two light guides. Fluoresced light from the heart was collected by a tandem lens assembly and directed to a dichroic mirror (560 nm, Omega Optical) that passes light of longer wavelengths to one 16×16-element photodiode array and reflects light of shorter wavelengths to a second 16×16-element photodiode array. Signals recorded from each photodiode and ECG signals were multiplexed and digitized with 12-bit precision at a sampling rate of 1000 Hz per channel (Microstar Laboratories Inc.). For the present study an optical magnification of ×1.24 was used, which resulted in a total mapping field of 1.4×1.4 cm with 0.09-cm spatial resolution. To view, digitize, and store the position of the mapping array relative to anatomic features, a mirror was temporarily inserted between the lenses of the tandem lens assembly to direct reflected light to a charge-coupled device video camera.

Experimental Protocol

The endocardial surface of the wedge preparation was stimulated at twice diastolic threshold current using a polytetrafluoroethylene-coated silver bipolar electrode with 1-mm interelectrode spacing. In a subset of experiments (n=4), pacing was performed from the epicardial surface. Calcium transient and action potential recordings were made during constant baseline pacing at 600 ms and during a momentary (5-second) step increase to the shortest cycle length that would capture the preparation 1:1 (~300 ms). The ECG, perfusion pressure, flow, and temperature were checked continuously throughout each experiment to monitor steady-state conditions. At the end of each experiment, tissue viability was confirmed using 10 mL of 2,3,5-triphenyltetrazolium chloride (TTC; 14 mg/mL) staining.

Data Analysis

Action potential depolarization and repolarization times were determined from the maximum first derivative during the upstroke and maximum second derivative during the repolarization phase, respectively. To quantify the rate of recovery of intracellular calcium to diastolic levels, the decay portion of the calcium transient (from 30% to 100% of the decline phase) was measured by the time constant (τ, Tau) of a single exponential fit, as previously used for calibrated and uncalibrated fluorescent signals. The duration of the calcium transient was also used to quantify calcium recovery and was defined as the difference in time from the onset of the calcium transient (20% above minimum diastolic level) to the point when intracellular calcium returns to within 90% of resting levels (CaF$_{90}$). To quantify calcium transient alternans (CaF ALT), the difference in the net amplitude of the larger and smaller calcium transient was expressed as a percentage of the net amplitude of the smaller transient. The increase in minimum diastolic calcium level during rapid pacing was measured as the difference in minimum diastolic level just before and after termination of rapid pacing and was expressed as a percentage of the net calcium transient amplitude during baseline pacing. All measurements were made using automated algorithms with visual inspection by an experienced investigator. Unless indicated otherwise, levels of significance were determined using a Student’s t test, where a value of P<0.05 was considered statistically significant.

Western Blot Analysis

Immediately after heart isolation (n=7), tissue samples (3.0×2.0×2.0 mm) weighing ~0.5 to 1.0 g were taken from the subepicardium, midmyocardium, and subendocardium. Samples were immediately frozen in liquid nitrogen and stored at ~80°C. Tissue samples were thawed, minced, and homogenized in 15 volumes of cold lysis buffer consisting of 1% Triton-X 100 and (in mmol/L) NaCl 150, Tris 50, and EDTA (pH 7.5) 1, containing freshly added protease inhibitors (Complete, Roche Molecular Biochemistry) and the phosphatase inhibitors sodium fluoride (50 mmol/L) and sodium orthovanadate (1 mmol/L). After 30 minutes on ice, insoluble debris was pelleted at 20 800g for 10 minutes at 4°C. Lysate protein concentrations were determined by the BCA method (Pierce), and aliquots were boiled in a reducing SDS sample buffer. Equal amounts of protein were subjected to SDS-PAGE and blotted to polyvinylidene difluoride membranes. After blocking overnight in 5% milk (Bio-Rad) in PBS-T (PBS plus 0.1% Tween-20) at 4°C, primary antibodies (monoclonal anti-SERCA2a, monoclonal anti-Na$^+$/Ca$^2+$ exchanger [Affinity Bioreagents] and monoclonal anti-actin [Sigma] as a control for protein loading) were diluted in blocking buffer and incubated with the blots for 1 hour at room temperature. Blots were washed with PBS-T, incubated with horseradish peroxidase–conjugated secondary antibody (Amersham Pharmacia) in blocking buffer for 1 hour at room temperature, washed again, and developed with the ECL-Plus kit (Amersham Pharmacia). Visualization and quantification of the bands were accomplished with the STORM phosphor imager loaded with ImageQuant software (Molecular Dynamics).

Results

Transmural Heterogeneity of Calcium Transients

Shown in Figure 1 is a picture of the wedge preparation (Figure 1A) with a representation of the mapping field superimposed (square). Shown below it are representative examples of calcium transients measured at a site near the endocardium (ENDO) and epicardium (EPI) under control conditions during steady-state endocardial pacing (cycle length 600 ms). At both sites, the calcium transient demonstrates a rapid increase in intracellular calcium followed by a slow decay as intracellular calcium returns to diastolic levels. When fit to a single exponential, the decay phase of the
calcium transient is slower (ie, Tau is larger) near the endocardium (250 ms) compared with the epicardium (135 ms). The slower decay of intracellular calcium near the endocardium is also evidenced by a longer calcium transient duration (480 ms) compared with the epicardium (400 ms). These data suggest that the decay of intracellular calcium to diastolic levels is slower at sites near the endocardium compared with the epicardium.

A transmural gradient in the decay of the calcium transient was evident throughout the entire mapping field. Shown in Figure 2 is a contour map of Tau (Figure 2A) that demonstrates a slower calcium transient decay (ie, larger Tau) closer to the endocardium compared with the epicardium throughout the mapping field. Similarly, CaF90 is longer near the endocardium (ENDO) compared with the epicardium (EPI). Furthermore, sites where Tau and CaF90 were slowest and longest, respectively, were not limited to the subendocardium and appear to extend into the midmyocardium. In a subset of experiments (n=4), pacing was performed from the epicardial surface, and Tau was also significantly slower (57%, P<0.04) near the endocardium (160±38 ms) compared with the epicardium (102±2 ms). These data suggest that the transmural heterogeneity of calcium handling is independent of activation sequence.

Transmural Heterogeneity of SERCA2a and Na\(^+\)-Ca\(^2+\) Exchanger (NCX) Expression

Tissue samples isolated from the subepicardial (EPI), midmyocardial (MID), and endocardial (ENDO) layers of the left ventricular wall demonstrate a transmural heterogeneity of SERCA2a and NCX protein levels. Shown in Figure 4 (top) are representative Western blots loaded with equal amounts of total protein. The subendocardial layer (ENDO) and midmyocardial layer (MID) showed less expression of
SERCA2a compared with the subepicardial layer (EPI). In contrast, a proteolytic fragment of NCX at 70 kDa appears equal across all layers. To account for variability across preparations, the average transmural expression of SERCA2a and NCX across all three layers was calculated for each experiment. Then, SERCA2a and NCX expression in each layer was calculated as a percentage of the average expression, respectively. Over all hearts, SERCA2a expression is significantly less (*P<0.02) in tissue samples taken from the subendocardial and midmyocardial layers compared with subepicardial layers. No significant difference in the transmural expression of NCX was observed. These data suggest that the transmural heterogeneity of Tau and CaF<sub>ALT</sub> as shown in Figure 3, may be due to transmural heterogeneity of SERCA2a expression.

Transmural Heterogeneity of CaF ALT

When the wedge preparation was paced rapidly, CaF ALT was observed. Shown in Figure 5 are calcium transients measured near the epicardium (EPI) and endocardium (ENDO) during an abrupt decrease in pacing cycle length from 600 to 300 ms. Immediately after rapid pacing begins, there is a transitory increase in calcium alternans near the endocardium and epicardium. Approximately 4 seconds after pacing cycle length was decreased, steady-state CaF ALT was observed, and CaF ALT levels for two consecutive beats (ie, beats a and b) were compared. Near the endocardium CaF ALT was 85%; however, near the epicardium CaF ALT was only 15%. The diastolic level of intracellular calcium preceding the small calcium transient (beat b) was always higher than that preceding the large calcium transient (beat a). Across all seven experiments, CaF ALT near the endocardium (101%±62%) was significantly greater (P<0.01) than at sites near the epicardium (12%±7%) for the same epicardial and endocardial sites used to compare Tau and CaF<sub>ALT</sub>. Alternans of the ECG T wave was also observed, which is consistent with repolarization alternans.

**Figure 3.** Shown for all experiments are Tau (top) and CaF<sub>ALT</sub> (bottom) from 8 equally spaced sites spanning the transmural wall during endocardial pacing. Data were normalized to fastest decay (ie, smallest Tau) and shortest duration, respectively, in each experiment. Tau and CaF<sub>ALT</sub> were significantly slower and longer (P<0.005), respectively, near the endocardium (ENDO) compared with the epicardium (EPI) over all experiments (n=7).

**Figure 4.** Representative Western blot (top) indicating expression of SERCA2a (110 kDa) and a proteolytic fragment of NCX (70 kDa) across the transmural wall, each loaded with equal amounts of protein. Subendocardial layers (ENDO) and midmyocardial layers (MID) showed less expression of SERCA2a compared with subepicardial layers (EPI). In contrast, no significant difference in transmural NCX expression was observed. To account for variability across preparations, the average expression of SERCA2a and NCX in all 3 layers was calculated for each heart (n=7). Then, SERCA2a and NCX expression in each layer was calculated as percentage of average expression. Over all hearts, only SERCA2a expression is significantly less (*P<0.02) in tissue samples taken from subendocardium (ENDO) and midmyocardium (MID) compared with subepicardium (EPI).

**Figure 5.** ECG and calcium transients near the epicardium (EPI) and endocardium (ENDO) recorded during an abrupt increase in pacing cycle length from 600 to 300 ms (arrow). Near ENDO and EPI, CaF ALT was persistent after 4 seconds of rapid pacing. At ENDO, where decay of the calcium transient was slower (174 ms) compared with EPI (93 ms), magnitude of CaF ALT was greater. At ENDO, calcium transient amplitude for beat a was 85% larger than that for beat b. In contrast, at EPI the degree of CaF ALT was less obvious (15%).
Shown in Figure 6A are calcium transients measured during rapid pacing, and calcium transients and action potentials measured during baseline pacing. We found that near the endocardium (left), CaF ALT and Tau are larger and slower compared with the epicardium (right). In contrast, action potential duration (APD) is slightly longer near the epicardium compared with the endocardium. Figure 6B shows a similar result across the entire mapping field. In this experiment, CaF ALT and Tau share a similar pattern, but APD does not. We found that across all experiments, APD near the endocardium (194±11 ms) was slightly longer than APD near the epicardium (187±23 ms) but, unlike Tau or CaF90, there was no statistical difference. APD restitution was assessed to determine whether repolarization alternans is responsible for CaF ALT. Shown in Figure 7 are graphs of APD as a function of diastolic interval for each beat after the step change in cycle length. APD decreased with diastolic interval; however, the slope of a linear fit is <1 near the endocardium (top) and epicardium (bottom). Over all experiments, the slope is <1, and there was no significant difference between the endocardium (0.64±0.09 ms) and epicardium (0.63±0.09 ms).

Transmurmal Heterogeneity of Diastolic Calcium Level During Rapid Pacing

The calcium transients shown in Figure 5 indicate an increase of diastolic intracellular calcium level during rapid pacing. In this example, the increase appears to be greater near the endocardium compared with the epicardium. This diastolic intracellular calcium increase was quantified in all experiments by measuring the difference between minimum diastolic levels just before and just after the termination of rapid pacing (Figure 8). Results from a different experiment (Figure 8A) demonstrate a similar finding in which the diastolic level of intracellular calcium near the endocardium (48%) was greater than that near the epicardium (23%). Across all experiments (Figure 8B), the diastolic intracellular calcium increase was significantly greater near the endocardium (41%±15%) compared with the epicardium (12%±14%, P<0.01).

Discussion

Transmurmal Heterogeneity of Calcium Handling

In this study, we found that endocardial cells exhibited a slower recovery of intracellular calcium to diastolic levels as measured by the exponential fit to the decay portion of the calcium transient (Tau) and the calcium transient duration (CaF90). Others have reported similar results from isolated cells 5,6 and the intact heart. It is important to note that in the present study calcium transient fluorescence was not calibrated, in part, because of problems associated with

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Figure 6. A, Calcium transient (CaF) and action potential (AP) recordings from endocardium (left) and epicardium (right) during rapid and baseline pacing. Near the endocardium, magnitude of alternans (CaF ALT) and decay of the calcium transient (Tau) were larger compared with the epicardium. In contrast, APD was slightly longer near the epicardium. B, Contour maps measured from a different experiment show similar results. Endocardial and epicardial surfaces (thin lines) are shown relative to the mapping field. Tau and APD were measured during baseline pacing, and CaF ALT was measured during rapid pacing. CaF ALT and Tau were larger and slower (dark gray), respectively, near the endocardial surface and shared a similar pattern. Pattern of APD, in contrast, was significantly different from that of CaF ALT and Tau.

Figure 7. Graphs of APD as a function of diastolic interval from the endocardium (ENDO) and epicardium (EPI) of the same preparation. APD and its preceding diastolic interval were determined for each beat after a step change in cycle length. APD decreased with diastolic interval; however, the slope of a linear fit to the data is <1 near the ENDO and EPI.
The sodium–calcium exchanger may also explain the SERCA2a expression near the endocardium in normal human hearts.5 The sodium-calcium exchanger is regional differences in the expression of calcium regulatory proteins. We measured a reduced molecular mechanism is regional differences in the expression of intracellular calcium (Ca\(^{2+}\)) increase during rapid pacing was measured as the difference between minimum diastolic level (dashed lines) just before and after termination of rapid pacing and was measured as a percentage of the calcium transient amplitude during steady-state baseline pacing. Diastolic CaF increase was greater near the ENDO (48%) compared with the EPI (23%). Over all experiments (B), the percentage diastolic CaF increase during rapid pacing was significantly (*P<0.01) greater near the endocardium than near the epicardium.

Figure 8. A, Calcium transients recorded from epicardium (EPI) and endocardium (ENDO) during the end of rapid pacing at a cycle length of 300 ms followed by the first beat of pacing at a cycle length of 1000 ms. The diastolic intracellular calcium (CaF) increase during rapid pacing was measured as the difference between minimum diastolic level (dashed lines) just before and after termination of rapid pacing and was measured as a percentage of the calcium transient amplitude during steady-state baseline pacing. Diastolic CaF increase was greater near the ENDO (48%) compared with the EPI (23%). Over all experiments (B), the percentage diastolic CaF increase during rapid pacing was significantly (*P<0.01) greater near the endocardium than near the epicardium.

ionophore-induced contracture. However, CaFalt, which is less dependent on the calibration of fluorescence, is consistent with Tau. Nevertheless, Tau must be interpreted with caution when considering specific calcium transport mechanisms.

The transmural heterogeneity of calcium handling we report is probably an intrinsic property of the cells, because activation sequence did not influence our result. One possible molecular mechanism is regional differences in the expression of calcium regulatory proteins. We measured a reduced expression of SERCA2a in the midmyocardial and endocardial layers compared with the epicardial layer. These data are consistent with previous studies that have shown significantly less Ca\(^{2+}\) ATPase mRNA in normal canine subendocardium compared with the subepicardium and a trend toward less SERCA2a expression near the endocardium in normal human hearts.5 The sodium-calcium exchanger may also explain the transmural heterogeneities of calcium handling that we observed. Recently, Zygmunt et al32 reported an unequal distribution of \(I_{\text{NaCa}}\) across the canine left ventricular wall, where peak reverse mode current is greatest in midmyocardial cells and weakest in endocardial cells. However, it is not clear whether forward-mode \(I_{\text{NaCa}}\) (ie, the removal of calcium) is also heterogeneous across the transmural wall. Our data suggest no significant difference in the expression of NCX across the transmural wall (Figure 4), and in normal human hearts mRNA levels of NCX1 are uniform across the transmural wall.5

Finally, because M cells extend to the deep subendocardium, it is also possible that prolonged APD associated with M cells increases intracellular calcium and thus prolongs the decay of intracellular calcium to diastolic levels.23 However, our studies were performed in intact preparations using a normal pacing cycle length (ie, 600 ms), when epicardial and endocardial differences in APD are less obvious.27–29 Indeed, over all experiments APD near the endocardium was only 7 ms longer than APD near the epicardium. Moreover, even though APD was slightly longer near the endocardium, the pattern of APD did not parallel the pattern of calcium transient decay (Figure 6B) and was variable across experiments. Therefore, it is difficult to explain the transmural heterogeneity of calcium handling we observed solely on the basis of on transmural differences in APD. The variability of transmural APD gradient that we measured may be due in part to small differences in transmural APD to begin with, wedge location,29 and the pattern of cell types across the transmural wall.27

CaF ALT During Rapid Pacing

The magnitude of pacing-induced CaF ALT was greater for cells near the endocardium compared with cells near the epicardium. CaF ALT has been reported previously in the whole heart,6,30–33 Interestingly, Wu and Clusin30 observed a larger level of alternans on the epicardial surface than in the present study using a similar method for calculating alternans. However, in that study alternans was observed in blood-perfused ischemic hearts. Given the close relationship between intracellular calcium and transmembrane potential, CaF ALT may be a mechanism of ECG T-wave alternans,11,34 Indeed, in our experiments CaF ALT was accompanied by T-wave alternans (Figure 5). Previous studies of acute myocardial infarction in canine have demonstrated a decrease in T-wave alternans and vulnerability to ventricular fibrillation with calcium channel blockers.35,36 These data are consistent with the findings of the present study and also suggest that the canine model is suitable for investigating the relationship between alternans and arrhythmia vulnerability.

The exact cellular mechanism of CaF ALT is not clearly understood. Slow cycling of SR calcium has been suggested as a mechanism of intracellular calcium alternans.8,9,37 In the rabbit heart the duration of the calcium transient increases during 2 to 3 minutes of ischemia, just as CaF ALT begins to develop.30 Our data show a high diastolic level of intracellular calcium followed by a small release of calcium from the SR and a low diastolic level of intracellular calcium followed by a large release of calcium from the SR. This suggests a close association between the recovery of intracellular calcium and the subsequent release of calcium from the SR.9,38 Notably, CaF ALT does not always occur when calcium recovery is delayed. For example, Figueredo et al6 observed reduced calcium handling near the endocardium during low-flow ischemia; however, no alternans was observed. This could be explained in part by differences in experimental conditions, species, or pacing rate. In addition, other possible mechanisms of CaF ALT may be related to modulation of SR release that is independent of calcium recovery.39,40 Finally, rather than heterogeneities of calcium cycling, it is possible that heterogeneities of APD restitution are responsible for APD and, thus, CaF ALT. The slope of the APD and diastolic interval relationship (ie, restitution) was shallow (ie, <1), and there was no significant difference between the endocardium and epicardium (Figure 7), which suggests that repolarization alternans is probably not responsible for CaF ALT. However, a traditional restitution protocol was not performed in this study, and therefore, these data must be interpreted with caution. A more systematic study of APD restitution, repolarization alternans, and calcium alternans is required.
**Increased Diastolic Level of Intracellular Calcium During Rapid Pacing**

In addition to CaF ALT, the apparent minimum diastolic level of intracellular calcium during rapid pacing was greater near the endocardium compared with the epicardium. Similar results have been reported in the rat heart and may occur because endocardial cells have a decreased ability to remove cytosolic calcium or have a greater calcium release from the SR (ie, CaF transient amplitude). In the present study we could not directly compare calcium transient amplitude; however, others have reported uniform calcium transient amplitudes from the epicardium and endocardium. Our results suggest that the higher level of diastolic intracellular calcium near the endocardium may be related to a slower decay of intracellular calcium caused by less SERCA2a. Interestingly, under normal conditions, we did not observe a progressive accumulation of intracellular calcium during rapid pacing. However, under abnormal conditions, such as ischemia, a progressive accumulation of intracellular calcium may occur preferentially near the endocardium and provide a substrate for arrhythmias.

It is possible that during rapid pacing the subendocardium is more susceptible to the subepicardium to demand-induced ischemia, which may be a mechanism for an increased level of diastolic intracellular calcium, slower calcium transient decline, or even CaF ALT. However, we believe this is unlikely in the present study because before, during, and immediately after rapid pacing there was no evidence of ischemia (triangulated action potentials or elevated/depressed ECG ST segment) and TTC staining showed no signs of chronic ischemia. Additionally, other studies have reported uniform transmural levels of NADH during normal coronary flow in the isolated heart and no evidence of ischemia in the wedge preparation. Alternatively, our data suggest that endocardial cells have an intrinsic decreased ability to remove cytosolic calcium because of a slower decay of intracellular calcium possibly caused by less SERCA2a. This heterogeneity of calcium handling may be a mechanism of CaF ALT and increased diastolic calcium levels during rapid pacing.

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**References**


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