Bradycardia alters Ca$^{2+}$ dynamics enhancing dispersion of repolarization and arrhythmia risk

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Submitted 22 October 2012; accepted in final form 21 December 2012

Kim JJ, Némec J, Papp R, Strongin R, Abramson JJ, Salama G. Bradycardia alters Ca$^{2+}$ dynamics enhancing dispersion of repolarization and arrhythmia risk. Am J Physiol Heart Circ Physiol 304: H848–H860, 2013. First published January 11, 2013; doi:10.1152/ajpheart.00787.2012.—Bradycardia prolongs action potential (AP) durations (APD adaptation), enhances dispersion of repolarization (DOR), and promotes tachyarrhythmias. Yet, the mechanisms responsible for enhanced DOR and tachyarrhythmias remain largely unexplored. Ca$^{2+}$ transients and APs were measured optically from Langendorff rabbit hearts at high (150 beats/min) or slow heart rate (SHR = 50 beats/min). Western blots and pharmacological interventions were used to elucidate the regional effects of bradycardia. As a result, bradycardia (SHR 50 beats/min) increased APDs gradually (time constant $\tau_{\text{f-s}} = 48 \pm 9.2$ s) and caused a secondary Ca$^{2+}$ release (SCR) from the sarcoplasmic reticulum during AP plateaus, occurring at the base on average of 184.4 ± 9.7 ms after the Ca$^{2+}$ transient upstroke. In subcellular imaging, SCRs were temporally synchronous and spatially homogeneous within myocardium. In diastole, SHR elicited variable asynchronous sarcoplasmic reticulum Ca$^{2+}$ release events leading to subcellular Ca$^{2+}$ waves, detectable only at high magnification. SCR was regionally heterogeneous, correlated with APD prolongation ($P < 0.01, n = 5$), enhanced DOR ($r = 0.9277 \pm 0.03, n = 7$), and was gradually reversed by pacing at 120 beats/min along with APD shortening ($P < 0.05, n = 5$). A stabilizer of leaky ryanodine receptors (RyR2), 3-(4-benzylcyclohexyl)-1-(7-methoxy-2,3-dihydrobenzo[f][1,4]thiazepin-4(5H)-yl)propan-1-one (K201; 1 µM), suppressed SCR and reduced APD at the base, thereby reducing DOR ($P < 0.02, n = 5$). Ventricular ectopy induced by bradycardia ($n = 5/15$) was suppressed by K201. Western blot analysis revealed spatial differences of voltage-gated L-type Ca$^{2+}$ channel protein (Cav1.2a), Na$^{+}$-$\text{Ca}^{2+}$ exchange (NCX1), voltage-gated Na$^{+}$ channel (Nav1.5), and rabbit ether-a-go-go-related (rERG) protein [but not RyR2 or sarcoplasmic reticulum Ca$^{2+}$ ATPase 2a] that correlate with the SCR distribution and explain the molecular basis for SCR heterogeneities. In conclusion, acute bradycardia elicits synchronized subcellular SCRs of sufficient magnitude to overcome the source-sink mismatch and to promote afterdepolarizations.

Bradycardia, defined as heart rate (HR) < 60 beats/min in adult humans (12), is usually a normal adaptive response to conditions of low metabolic demand, such as rest and sleep. Bradycardia due to pathological conditions, e.g., sick sinus syndrome, atrioventricular (AV) nodal disease, or conduction system disease, is common in clinical practice and frequently causes symptoms because of inappropriately low cardiac output. Less frequently, profound bradycardia, usually caused by heart block, may paradoxically cause ventricular tachycardia, often polymorphic ventricular tachycardia indistinguishable from Torsades de Pointes (TdP) that is classically observed in acquired or congenital long QT syndrome (LQTS) (57).

Bradycardia is known to be a cofactor in the initiation of LQT-related arrhythmias in humans and most animal models of LQTS (56, 58); ventricular pacing at relatively high rates is an effective treatment of TdP. Occasionally, severe bradycardia appears to be the only trigger of TdP in patients lacking other obvious preexisting causes of repolarization delay. Polymorphic ventricular tachycardia may also occur as a consequence of relative bradycardia after a prolonged tachycardia, e.g., following radiofrequency ablation of the AV node for atrial fibrillation or ablation of an accessory pathway causing incessant tachycardia (14, 15, 25). This potentially fatal complication occurs frequently enough that pacing at relatively high rates (80–90 beats/min) early after radiofrequency ablation of AV node is recommended (39).

Bradycardia-dependent TdP is more prevalent in women (~75%) than men (54, 57), which parallels the well-established finding for drug-induced LQT type 2 (LQT2). On the other hand, most episodes of TdP in humans are not preceded by profound bradycardia, and more than one factor is often identified (29, 32).

Effects of bradycardia on action potential duration and Ca$^{2+}$ handling. Action potential (AP) duration (APD) adaptation to HR changes is required to maintain an adequate ratio between ventricular filling and ventricular ejection. The mechanisms responsible for APD adaptation have been the topic of extensive investigation. Most studies attributed rate-dependent APD adaptation to changes in intracellular Ca$^{2+}$ (Ca$^{2+}$), which in turn alters the kinetics of Ca$^{2+}$-dependent inactivation of L-type Ca$^{2+}$ channels (4, 9, 41). During a transition from slow to fast HR, diastolic Ca$^{2+}$ rises because of higher influx (a consequence of a greater number of APs per unit time) and decreased efflux because of shorter diastolic intervals. Higher Ca$^{2+}$ accelerates the Ca$^{2+}$-dependent inactivation of L-type Ca$^{2+}$ currents ($I_{\text{Ca,L}}$) and reduces Ca$^{2+}$ influx per AP, which lowers the plateau phase and decreases APD. Others have implicated the Na$^{+}$-$\text{Ca}^{2+}$ exchange (NCX) current ($I_{\text{NCX}}$) (21) and the late Na$^{+}$ current ($I_{\text{Na,L}}$) (24). Mathematical simulations of slow HR (SHR) indicated that the long diastolic intervals result in a complete deactivation of the slow component of the delayed rectifying K$^{+}$ current ($I_{\text{Kd}}$) and complete recovery from inactivation of $I_{\text{Ca,L}}$, which could theoretically explain APD adaptation in bradycardia (5, 27, 61). Besides ion channel kinetics, changes in tonic concentrations in the cytoplasm...
(intracellular Na\(^+\) and Ca\(^+\)) or extracellular K\(^+\) may contribute to APD adaptation (13, 22).

**HR and arrhythmias.** HRs outside the physiological range may promote arrhythmias through changes in Ca\(^+\)\(^+\) handling (45). Transitions from slow to fast HR have been extensively studied and shown to create Ca\(^+\)\(^+\) alternans that lead to APD and T-wave alternans, increased dispersion of repolarization (DOR), and arrhythmias (42). In the setting of ischemia (43, 44) and heart failure (64), arrhythmogenic Ca\(^+\)\(^+\) (DOR), and arrhythmias (42). In the setting of ischemia (43, 44) and heart failure (64), arrhythmogenic Ca\(^+\)\(^+\) (DOR), and arrhythmias (42).

SHR promotes sarcoplasmic reticulum (SR) Ca\(^+\)\(^+\) overload, resulting in spontaneous SR Ca\(^+\)\(^+\) release during the AP plateau and early afterdepolarizations (EADs) (18, 38). In intact hearts, bradycardia prolongs APD and enhances DOR (16, 60), which are thought to contribute to LQT2-related arrhythmias.

This project investigates APD adaptation and Ca\(^+\)\(^+\) handling changes during bradycardia in Langendorff-perfused rabbit hearts at low and high magnifications. We show that bradycardia promotes a secondary intracellular Ca\(^+\)\(^+\) (Ca\(_i\)) release at the base of the ventricles, which contributes to further APD prolongation, increased DOR, and triggered activity.

**METHODS**

**Heart preparations.** New Zealand White rabbits (females, 60 to 120 days old) were euthanized with pentobarbital sodium (75 mg/kg intravenously) and anticoagulated with heparin (200 U/kg intravenously). The heart was rapidly dissected and perfused with Tyrode solution containing (in mM) 130 NaCl, 24 NaHCO\(_3\), 1.0 MgCl\(_2\), 4 KCl, 1.2 NaH\(_2\)PO\(_4\), 50 dextrose, and 1.25 CaCl\(_2\) (at pH 7.2–7.4), gassed with 95% O\(_2\) plus 5% CO\(_2\). Temperature and perfusion pressure were continuously monitored and regulated by a feedback system at 37 ± 2°C and ~80 mmHg, respectively. To minimize motion artifact, blebbistatin (5–10 μM; Sigma, St Louis, MO) was added to the perfusate for 5–10 min. The heart was immobilized in a chamber and stained with a voltage-sensitive dye (PGH1: 200 μl of 1 mg/ml dimethyl sulfoxide) and loaded with a Ca\(^+\)\(^+\) indicator (Rhod-2 AM, 200 μl of 1 mg/ml dimethyl sulfoxide). Epicardial bipolar pseudo-EKG was continuously monitored, and the atrioventricular (AV) node was ablated to control HR. Hearts were paced with an epicardial unipolar electrode placed on the lateral wall of the right ventricle, approximately halfway between the apex and base. In pilot experiments, different pacing sites at the apex, base, and posterior were tested and found not to alter the effect of bradycardia on Ca\(^+\)\(^+\) dynamics. For a physiological rate, hearts were paced at a baseline cycle length of 0.5 s [baseline HR (BHR) of 120 beats/min] and for bradycardia at a SHR with a cycle length of 1.2 s (50 beats/min; a profound bradycardia for rabbit hearts). This investigation conformed to the current Guide for Care and Use of Laboratory Animals, published by the National Institutes of Health, and was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

**Optical apparatus.** An optical apparatus consisting of two CMOS cameras (SciMedia, Ultima One, 100 × 100 pixels) has been used for simultaneous measurement of intracellular Ca\(^+\)\(^+\) transients (CaTs) and membrane potential (V\(_m\)) changes, as previously described (47). For long uninterrupted recordings at low magnification, a slow scan rate of 250 frames/s was intentionally chosen to record Ca\(_i\) and V\(_m\) during two to three sets of transitions from BHR to SHR and SHR to BHR. Brief (4 s) recordings were taken at 2,000 frames/s to verify the kinetics of Ca\(_i\) and V\(_m\) upstrokes. Subcellular Ca\(_i\) and V\(_m\) signals were measured at high magnification at scan rates of 200 frames/s to achieve a high signal-to-noise ratio without compromising the kinetics of the signals. The anterior surface of the heart was illuminated with a 520 ± 30-nm excitation beam, and the fluorescence emitted by Rhod-2 and PGH1 was separated by a dichroic mirror (660 nm) and was focused on two CMOS cameras, as previously described (47). Some hearts were stained with 4-[(β-[2-(di-n-butylamino)-6-naphthyl]vinyl)pyridinium (di-4-ANEPBP2 to better visualize transverse tubules at high optical magnification.

**Macroscopic and subcellular Ca\(_i\) and V\(_m\) mapping.** For macroscopic imaging, each camera viewed 1.5 × 1.5 cm\(^2\) from the anterior

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**Fig. 1.** Action potential (AP) duration (APD) adaptation during transitions from baseline heart rate (BHR) and slow heart rate (SHR). APD\(_{80}\) was calculated from the interval between the maximum first derivative of the AP to 20% of the APD (b) as shown uninterrupted for 2,200 s. B: optical traces of APs from a single pixel acquired at low magnification at time points a–e, as labeled in A. top trace. C: near-field EKG signals recorded from the left ventricular epicardium at the same time points as depicted in A, top trace. The relative amplitude of T waves (arrows) measured as the ratio of T wave to QRS amplitudes was significantly higher in SHR than BHR, which is indicative of an increase in dispersion of repolarization.
surface of the heart using a Nikon camera lens (50 mm, 1:1.2). For subcellular imaging, the heart was perfused horizontally in a chamber on the stage of an upright microscope (Olympus BX61W1) using a ×40 water immersion objective (Olympus, ×40/LUMPLFL). Fluorescence microscopy to image intact hearts at the subcellular level offers important advantages compared with previous confocal imaging studies (2, 50). The lack of confocal apertures results in considerably greater light throughput (2 to 3 orders in magnitude), making it possible to measure Cai and Vm signals from two-dimensional fields of view (instead of line scans) with a greater signal-to-noise ratio (>80/1) and temporal resolution (up to 2,000 frames/s). Butterworth low-pass temporal filtering (60- to 100-Hz cutoff) was used if needed, but spatial filtering was not applied to maximize spatial resolution. The limitation of bright field over confocal microscopy is the blurring caused by background scattered light from deeper layers of muscle and the lower voxel resolution. In the present configuration, the ×40 objective has a depth of field of ~0.5 μm and an x-y resolution of 1.5 × 1.5 μm². The confocal apertures would block scattered light emanating from out-of-focus fluorescence sources and would improve contrast by reducing blurring. More relevant to this study, nonconfocal optics does not significantly alter Cai, kinetics in maps of subcellular Ca²⁺ waves in three dimensions. A recent analysis of spark properties recorded with confocal imaging revealed that the spatial widths and rise times of sparks were similar when recorded in-focus compared with out-of-focus by ±1 μm (51). We estimated that imaging with a ×40 objective represented the summation of Cai, or (Vm) signals over a voxel (1.5 × 1.5 × 3.0 μm³) with reduced spatial but an improved temporal resolution compared with confocal microscopy.

Study protocol. The AV node was ablated by cautereization to allow control of HR by pacing. Hearts were paced at a cycle length of 0.5 s, which was taken as a BHR, and the cycle length was then lengthened to 1.2 s to impose a bradycardia or a SHR. APs and CaTs were continuously recorded during several (typically 3) transitions from fast to slow and back from slow to fast HR. Changes in HR led to gradual changes in APD until a new steady state was attained. The time course of APD adaptation required continuous recordings of 3–5 min to reach a steady state of APDs during transitions from BHR to SHR (bradycardia) and 5–10 min in going from SHR to BHR.

Pilot experiments were carried out to select the BHR and the SHR that were used in this study. A SHR of 50 beats/min was chosen because it could be maintained reliably without interruptions by an occasional extra beat yet was effective to expose spatial heterogeneities of CaT caused by secondary Ca²⁺ release (SCR). Similar, but less pronounced, effects were observed at 0.9- and 1.0-s cycle lengths. A basic HR of 120 beats/min was chosen because 1) this HR was well tolerated and did not result in rundown of the preparations, 2) capture with pacing electrodes was reliable during uninterrupted recordings, and 3) spatial heterogeneities of AP and CaT were negligible compared with still faster rates.

Fig. 2. Time course of APD and Ca²⁺ transient (CaT) duration (CaTD) adaptation. A: changes in AP and CaT dynamics recorded at low magnification during a change in HR from BHR to SHR (top traces). CaTD and APD are plotted as a function of time (bottom left and bottom middle, respectively), and APD vs. CaTD reveals a tight linearly relationship (bottom right). Cai, intracellular Ca²⁺. Inset: superposition of APs and CaTs from the same pixel on the RVB recorded at different times during adaptation to SHR; arrow points toward steady-state SHR. B: changes in AP and CaT dynamics during the transition from SHR to BHR. APD and CaTD are plotted as a function of time (bottom left and bottom middle, respectively) and APD vs. CaTD (bottom right) are linearly related. Inset: superposition of APs and CaTs from the same pixel recorded at different times during adaptation toward BHR; arrow points toward steady-state BHR.
**Data analysis.** Activation time at each site was calculated from maximum first derivative of the fluorescent signal \[(dF/dt)_{\text{max}}\] of the local AP or CaT upstroke. APD and CaT duration (CaTD) at each site were calculated from the interval between \((dF/dt)_{\text{max}}\) and the recovery of \(V_o\), and CaT traces to 20% of baseline (APD\(_{80}\) or CaTD\(_{80}\)), respectively. Automatic measurement of APD\(_{80}\) and CaTD\(_{80}\) from all pixels (100 \times 100 pixels) was used to calculate mean APD\(_{80}\) and CaTD\(_{80}\). The dispersion of APD\(_{80}\) was calculated from the SD of APD\(_{80}\), the rate constant \((\tau)\) of mean APD from all 100 \times 100 pixels) was used to calculate mean APD\(_{80}\) and CaTD\(_{80}\). The dispersion of APD\(_{80}\) was calculated from the SD of APD\(_{80}\). The time constant \((\tau)\) of mean APD (from all 100 \times 100 pixels) adaptation to HR changes was calculated by fitting the transitions from BHR to SHR \((\tau_{BHR}\rightarrow SHR)\) and from SHR to BHR \((\tau_{SHR}\rightarrow BHR)\) with monoeXponential functions. Rate and time-dependent mean APD\(_{80}\) and regional variation in 100 \times 100 pixels) adaptation to HR changes was calculated by fitting the transition of CaTD\(_{80}\) at each pixel. All 10,000 recordings (100 \times 100 pixels) were used to generate the scatterplots to correlate spatial heterogeneities of ASCRs to the dispersion of APD\(_{80}\). Regional differences of APD\(_{80}\), base of right ventricle (RVB), and apex of left ventricle (LVA) were compared for statistical significance using two-tailed t-test. Box-whisker diagrams are used to visualize the distribution of the data. The top and bottom whiskers define, respectively, the maximum and minimum values; the top and bottom of the box define the 75th and 25th percentiles, respectively, and the line within the box is the median in the data set.

**Western blot analysis.** Female New Zealand White rabbits (3 mo old) were euthanized as described above, the hearts were perfused with Tyrode solution, and ventricular tissue samples (~50 mg) were dissected from the base (B) and apex (A) of the epicardium. Proteins were isolated as previously described (17), were separated by SDS-PAGE (50 µg/sample) and transferred to polyvinylidene difluoride membranes, which were probed by standard techniques. After immunolabeling, band intensities were measured with ImageJ and normal-
Bradycardia-induced changes in CaT. Low-resolution imaging of V_m and Ca signals during HR transition was performed to confirm the reproducibility of rate-dependent changes. APD_{80} adaptation was reproducible from heart to heart and during repeated cycles (3 to 4 per heart) of transitions from 120 (BHR) to 50 (SHR) beats/min and back. Figure 1A illustrates a continuous sequence of two complete cycles of APD adaptation from BHR to SHR. From a HR of 120 beats/min (not shown), a transition to SHR resulted in a gradual prolongation of APD_{80} and QT interval which was fully reversed by pacing at a BHR. The mean ΔAPD_{80} in the first episode of SHR increased by 55.2 ± 10.9%, which was similar to that measured in a second episode of SHR (51.25 ± 8.2%) (P < 0.05, n = 4). Fig. 1, B and C, illustrates optical APs and EKG recordings measured at time points a–e (Fig. 1A). An AP recorded at the onset SHR (trace a) is markedly shorter than at steady-state SHR (trace b). APs measured at the next BHR are shorter than at steady-state SHR. Similarly, T-wave amplitudes were markedly larger at SHR than at BHR or the initiation of SHR (Fig. 1C). The changes in T-wave amplitude relative to the QRS amplitude are a measure of changed repolarization gradient and the ratio of T wave to QRS amplitude was statistically greater at steady-state SHR than BHR (Fig. 1C, right, P < 0.01, n = 15 trials from 5 hearts).

At BHR, the time course of AP and CaT signals exhibited the expected rapid rise and monophasic recovery to baseline. After the transition to SHR, diastolic levels of Ca_i decreased gradually and APD_{80} and CaT_{80} exhibited the expected time-dependent prolongation (Fig. 2A). Most interesting was the gradual prolongation of CaT_{80}, which was associated with a slowing down of the CaT downstroke, the appearance of a Ca_i plateau, and a small secondary Ca^{2+} peak. This increasingly more pronounced SCR during the AP plateau was associated with APD prolongation, changes in the shape of APs,
with a tight linear correlation between CaTD\textsubscript{80} and APD\textsubscript{80} (Fig. 2A). Reversal to BHR suppressed SCR gradually, altered the shape of AP repolarization, and shortened APD\textsubscript{80} and CaTD\textsubscript{80} (Fig. 2B). APD\textsubscript{80} adaptation curves exhibited monoexponential time courses with significantly different time constants in going from BHR to SHR (\(\tau_{\text{f-s}} = 48 \pm 9.2\) s) compared with from SHR to BHR (\(\tau_{\text{f-s}} = 30.4 \pm 4.7\) s; \(P < 0.05, n = 5\) hearts). A similar trend was reported for QT adaptation in patients upon sudden HR changes (49).

**Regional heterogeneity of SCR.** We calculated the spatial correlation between changes in AP and CaT to evaluate the interplay between SCR and \(V_m\) and Ca\textsubscript{a}. The distribution of SCR was heterogeneous, being more pronounced at the RVB than the LVA. The superposition of CaT measured at BHR and 5 min of SHR exemplify the heterogeneous changes that occur from RVB to LVA (\(a, b,\) and \(c\)) on the epicardium (Fig. 3, \(A\) and \(B\)). The delay between the first and second release of Ca\textsuperscript{2+} (SCR) was measured from the time interval between (\(d^2F/dt^2\)\textsubscript{max} and the inflection point (\(d^2F/dt^2 = 0\)) of the CaT downstroke. When measured for all 10,000 pixels in 5 hearts, SCR occurred 184.4 \(\pm\) 9.7 ms after the first Ca\textsuperscript{2+} release, demonstrating a highly reproducible change in Ca\textsuperscript{2+} dynamics at all sites on the base of the epicardium and from heart to heart. Similarly, the ASCR was assessed by measuring the area under the CaT curve between the inflection point (\(d^2F/dt^2 = 0\)) and CaTD\textsubscript{80}.

The interplay between SCR and \(V_m\) was evaluated by correlating ASCR with APD\textsubscript{80}. Maps of APD\textsubscript{80} and ASCRs are shown for BHR and after 5 min of SHR, which increased the dispersion of APD\textsubscript{80} and ASCRs (\(P < 0.01, n = 7\) hearts) (Fig. 3, \(C\) and \(D\)). Most interesting was the statistical analysis that revealed an enhanced correlation between ASCRs and APD\textsubscript{80} (\(r = 0.90 \pm 0.03\)) during SHR compared with BHR (\(r = 0.66 \pm 0.18\) (\(P < 0.01, n = 7\) hearts) (Fig. 3E)). SHR significantly increased the dispersion of APD\textsubscript{80} compared with BHR (\(P < 0.01, n = 7\); Fig. 3F), and mean APD\textsubscript{80} (averaged over 24 pixels) was significantly longer at the RVB than the LVA (\(P < 0.05, n = 7\), Fig. 3G).

**Subcellular imaging.** Hearts were mapped at high magnification to elucidate the cellular mechanisms underlying heterogeneities of SCR; this visualized CaT changes in unprecedented detail. When stained with di-4-ANEPPS, the transverse tubules are readily visualized with nonconfocal imaging (Fig. 4A). When stained with PGH1 and Rhod-2 AM, individual myocytes can be identified and subcellular recordings of APs and CaT were measured from a single pixel viewing 1.5 \(\times\) 1.5 \(\mu m^2\) and a signal-to-noise ratio > 80/1 (Fig. 4B). Changes in CaT were analyzed by converting our two-dimensional data (Fig. 4C, see Fig. 5. SHR promotes subcellular SCRs and CaT prolongation at the base but not the apex. A: locations of sites 1 and 2 (RVB and LVA, respectively) is indicated on this photograph of anterior wall of the heart. The high-magnification tracings from these sites are displayed in the other panels. B: subcellular image taken with the \(\times 40\) objective from site 1 at the RVB, as indicated in A. C: Ca\textsubscript{a} transients recorded along line \(a-a'\) in RVB shown in B at different HR in the line scan format along with tracings from the pixel located in the middle of line \(a-a'\). Note the increasingly more pronounced SCR in SHR. D: Ca\textsubscript{a} transients recorded along the longitudinal axis of a fiber in LVA at SHR.
important ways: the systolic Ca\textsuperscript{i} exhibited a SCR and diastolic Ca\textsuperscript{i} exhibited subcellular oscillations that were not apparent at low magnification (Fig. 4D, a). After 5 min at a SHR, the line scans changed in two important ways: the systolic Ca\textsuperscript{i} exhibited a SCR and diastolic Ca\textsuperscript{i} exhibited subcellular oscillations that were not apparent at low magnification (Fig. 4D, b). Reverting to the BHR for 5 min fully reversed these changes in subcellular Ca\textsuperscript{i} (Fig. 4D, c). These subcellular changes in Ca\textsuperscript{i} handling seen at SHR were highly reproducible within the same heart and were reproduced in five hearts (Fig. 4E). As illustrated in Fig. 5, SCRs increased in amplitude as a function of time at SHR and were considerably more pronounced at the base than at the apex. Note the absence of SCR at a site on the apex (Fig. 5D), whereas a well-developed SCR was present at basal site (Fig. 5C).

The significance of SCR in bradycardia was investigated with respect to spatial and temporal synchrony among cells at the base of the heart. Figure 6 illustrates the changes in CaT in bradycardia in a two-dimensional recording as a movie (Fig. 6A, see supplementary movie 2) and as line scans along the longitudinal (Fig. 6B, top, along a–a’) and transverse (Fig. 6B, bottom, along b–b’) axis of a myocyte. The video clip shows that SCR occurs synchronously within each cell (<20 ms) and between adjacent cells although with different amplitudes in various cells. In contrast, diastolic Ca\textsuperscript{2+} waves are temporally and spatially asynchronous and did not propagate across cell borders. An activation map of SCR within a cell showed that SCR was highly synchronous (Fig. 6C, top), whereas Ca\textsuperscript{2+} waves during diastole propagated slowly and were random within cells (Fig. 6C, bottom). Superimposed Ca\textsuperscript{i} and V\textsubscript{m} signals from a 10.5 × 10.5 μm\textsuperscript{2} region of a single myocyte are shown in Fig. 6D. The onset of systolic SCR corresponds to change in AP slope. Diastolic Ca\textsuperscript{i} rise and corresponding depolarization are present in the same cell region. Interestingly,
the amplitude of systolic SCRs and the diastolic Ca waves in a given cell was tightly correlated, suggesting a related mechanism \((r = 0.86 \pm 0.021, n = 4 \text{ hearts}; \text{Fig. 6E}).\)

**Mechanism of SCR.** To further elucidate the interplay between Ca, and voltage, experiments were carried out to assess whether SCR prolonged APD \(_{80}\) or longer APD \(_{80}\) elicited SCRs. In SHR, it is reasonable to expect that SCRs originate from SR \(\text{Ca}^{2+}\) release via RyR2 because the rise of SCRs always preceded those of partial depolarizations determined from inflection points of AP during the AP plateau (or late-phase voltage humps) by 6.4 \pm 1.3 ms. An agent known to stabilize RyR2, K201 (1 \(\mu\text{M}, n = 5\)) was tested for its effects on SCR. Plots of mean \(\Delta\text{APD}_{80}\) as a function of time are shown during transitions from BHR to SHR from 5 \times 5 pixels on the LVA (Fig. 7A, top) and 5 \times 5 pixels on the RVB (Fig. 7A, bottom) with and without K201. The graphs show that K201 reduced mean APD \(_{80}\) at pixels on the RVB (\(P < 0.01, n = 5\)) but did not significantly alter AP and CaT at the apex. K201 did not alter diastolic Ca\(^{2+}\) levels or the amplitude of CaTs. In the absence of K201 baseline drift recorded a percent change of fluorescence relative to the amplitude of CaT was \(-1.02 \pm 0.26\%\) per min, and in the presence of K201, the slope was \(-1.14 \pm 0.16\%\) per min \((P = 0.19, n = 5 \text{ hearts}).\) Similarly, the fractional change of fluorescence during a CaT, fractional fluorescence change \((\Delta F/F)\) was 0.434 \pm 0.11 in the absence and 0.442 \pm 0.126 in the presence of K201 \((P = 0.24, n = 5).\)

K201 did not completely abolish APD adaptation but suppressed the difference in APD prolongation between apex and base. As shown in Fig. 7B, the suppression of SCR by K201 markedly reduced the dispersion of APD \(_{80}\) and DOR at SHR \((P < 0.05, n = 5).\)

The molecular basis underlying the higher occurrence of SCR and diastolic \(\text{Ca}^{2+}\) waves at the base compared with the apex is most likely due to heterogeneities in the expression of ion channels and transporters. As shown in Fig. 8, the expression of the \(\alpha\)-subunit of voltage-gated L-type \(\text{Ca}^{2+}\) channel protein, Cav1.2; the predominant NCX protein found in the heart, NCX1, and voltage-gated Na\(^{+}\) channel, Nav1.5 proteins was significantly higher at the base (B) than the apex (A) \((P < 0.05; n = 7).\) In contrast, the levels of SERCA2A and RyR2 were not significantly different and RERG had the opposite distribution, being higher at the apex than the base \((P < 0.05; n = 6 \text{ hearts}).\)

**Role of SCR in bradycardia-induced ventricular ectopy.** We performed a systematic evaluation of relationship between SCR and ventricular ectopy. In 5 out of 15 hearts studied at low optical magnification, bradycardia alone was sufficient to produce premature ectopic beats that were eliminated by pacing at BHR or by inhibiting SCR with K201. In the five hearts that developed ectopic beats during bradycardia, ectopic beats appeared after a few minutes (Fig. 9, B and D) and were readily abolished by reverting back to BHR (Fig. 9, C and E). The addition of K201 had no effect on APs and CaT in BHR but blocked ectopic activity after 5 min of SHR (Fig. 9F). The kinetics of AP and CaT upstrokes were analyzed for paced (black arrows) and ectopic beats (gray arrows; Fig. 9G). Activation maps of the paced beats and ectopic beats show that paced beats were initiated at the location of the stimulus electrode and ectopic beats emanated from the RVB, leading to a wavefront that collided with the paced wavefront (Fig. 9H). For all paced beats, \(V_{m}\) preceded Ca\(_{i}\) (not shown) but Ca\(_{i}\) preceded \(V_{m}\) for all ectopic beats at their origins, located at the base and most often on the RVB (Fig. 9, H and I, site a). However, as the ectopic beat propagates away from its origin, \(V_{m}\) preceded Ca\(_{i}\) (site b; Fig. 9, H and I).

**DISCUSSION**

HR is an important mechanism used by mammals to adjust cardiac output to changing demands. The adaptive role of
tachycardia in a fight-or-flight situation is well accepted, as is the diminished myocardial energy consumption during bradycardia. Several parameters of cardiac contraction have to adjust to dynamic HR changes. At the cellular level, this requires an adaptation of APD and CaT in response to a change in HR.

This study showed that bradycardia changes in Ca\(^{2+}\) dynamics are spatially heterogeneous, being more pronounced at the base of the ventricles. Bradycardia elicits an SCR occurring during the AP plateau, which are synchronous within a given cell and within the myocytes in the high-power field of view. It also leads to diastolic SR Ca\(^{2+}\) release in the form of propagated Ca\(^{2+}\) waves, which are not synchronized among adjacent cells. Ca\(^{2+}\) imaging at low magnification demonstrated a tight correlation of SCR with enhanced APD. Interestingly, high-magnification Ca\(^{2+}\) imaging was required for detection of diastolic Ca\(^{2+}\) waves, since their detection was prevented by their lack of tight temporal synchronization and by spatial averaging over the multiple myocytes comprising a low-magnification pixel.

It is possible that the higher expression of Cav1.2α and NCX1 at the base of the heart reported here and elsewhere may partially explain the spatial heterogeneity of Ca\(^{2+}\) handling during bradycardia, e.g., by causing a higher degree of SR load at the ventricular base.

In principle, ectopic activity induced by bradycardia could be attributed to a spontaneous reactivation of I\(_{Ca-L}\), independent of altered Ca\(^{2+}\) dynamics. Alternatively, bradycardia could promote SR Ca\(^{2+}\) overload and produce SCR that activates a depolarizing I\(_{SCX}\), with I\(_{NCX}\) causing the initial depolarization, which results in I\(_{Ca-L}\) reactivation. In our opinion, compelling evidence implicates SCR as the initial trigger of EADs and ectopic activity in bradycardia. First, SCR frequently occurred with no apparent voltage deflection, and when EADs were present, the SCR onset preceded EAD onset by 5–10 ms at the site of the ectopic focus. Second, pharmacological intervention with K201, a RyR2 stabilizer, suppressed SCR, DOR, EADs, and ectopic activity, yet had no significant effect on APD and CaT at the apex or sites that had no SCR. The effect of K201 on SCR could not be attributed to an indirect effect on Ca\(^{2+}\) load, since K201 did not alter baseline Ca\(^{2+}\) compared with controls. Given the stability and signal quality of Ca\(^{2+}\) measurements, K201 did not alter diastolic Ca\(^{2+}\).

**Triggered activity in bradycardia and impaired repolarization.** Slow ventricular rate is an established proarrhythmic factor known to increase the propensity to TdP, an arrhythmia associated with delayed repolarization (40, 54). Abnormal Ca\(^{2+}\) handling and spontaneous SR Ca\(^{2+}\) release has been suggested as the mechanism of EADs, the form of triggered activity which underlies TdP. (18, 55, 62) We have recently reported that in rabbit hearts with LQT2, TdP elicited by bradycardia and delayed rectifying K\(^+\) current (I\(_{Kr}\)) blockade exhibited Ca\(^{2+}\) oscillations during APD prolongation that preceded the appearance of EADs by minutes (38). The data suggest that Ca\(^{2+}\) oscillations are caused by spontaneous SR Ca\(^{2+}\) release that activates a depolarizing I\(_{SCX}\) that serves as a trigger to EAD generation. This study was motivated by the need to elucidate the relationship between bradycardia, Ca\(^{2+}\) handling and arrhythmogenesis. We observed that bradycardia alone caused a lesser degree of SCR, sometimes manifested as delayed CaT downstroke rather than CaT reelevation. This could facilitate the generation of EADs when repolarization is impaired and occasionally suffice to trigger EADs on its own.

Bradycardia has long been known to prolong APDs and enhance DOR, setting the stage for functional reentry, but the mechanisms that enhance APD and DOR are not well understood. Here, we report that bradycardia produces the expected gradual increase of APDs and decrease of diastolic Ca\(^{2+}\). SCR contributes to spatial heterogeneities of APD and enhanced DOR, since its elimination reduces DOR.

**Mechanism underlying SCR.** The mechanism whereby bradycardia promotes SCR is uncertain at this moment. In principle, increased diastolic interval during bradycardia should allow more time for Ca\(^{2+}\) removal out of the cell by NCX. However, SR Ca\(^{2+}\) pumps can effectively compete with NCX...
for the removal of $\text{Ca}_{\text{ii}}$ during bradycardia. The amount of $\text{Ca}_{\text{ii}}$ transported to the lumen of the SR by SERCA during each cardiac cycle may actually be higher during bradycardia due to 1) longer AP plateau (perhaps a contribution of reduced $I_{\text{Kr}}$ at the base), 2) longer duration of $\text{Ca}^{2+}$ influx through L-type channels, and 3) the reduced NCX driving force during more positive plateau potentials. If there is a significant diffusion limitation of $\text{Ca}^{2+}$ movement between the uptake and release compartments of the SR, the functional SR may be replenished after the initial phase of $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$-release from the nonjunctional compartment during each heartbeat to a degree which allows spontaneous $\text{Ca}^{2+}$ release through RyR2 in at least some myocytes. This process should be augmented by any intervention which prolongs plateau duration, such as $I_{\text{Kr}}$ blockade.

$\text{Ca}_{\text{ii}}$ imaging shows that SCR occurs uniformly within each cell and is temporally synchronous among neighboring cells despite cell-to-cell differences in ASCR. The temporal synchronization can be attributed to similar time needed to overload the lumen of the SR network during long APDs due to incomplete voltage-dependent inactivation of L-type $\text{Ca}^{2+}$ channels.

It is likely that SR loading is accentuated in regions with increased $I_{\text{Ca,L}}$ density. This is consistent with the observation of SCR occurring preferentially at basal regions of ventricular myocardium. We previously reported that female rabbit hearts express higher protein levels of Cav1.2x and NCX1 and have significantly higher current densities of $I_{\text{Ca,L}}$ and $I_{\text{NCX}}$ (25–30%) at the base compared with the apex. The greater $\text{Ca}^{2+}$ influx via $I_{\text{Ca,L}}$ and reverse mode $I_{\text{NCX}}$ lead to greater SR $\text{Ca}^{2+}$ overload and a greater propensity to EADs in female myocytes isolated from the base compared with those isolated from the apex, endocardium or isolated from male hearts (17, 52, 65).

**SCR prolong APD.** While AP prolongation caused by bradycardia may be the primary cause for SCR development, it appears likely that once SCRs occur, they may themselves prolong APD. We suppressed SCR with K201 to confirm their causative effect on APD prolongation. K201 has been found to alter the stabilized state of cardiac RyR (26, 33) and suppress the nonvoltage-triggered $\text{Ca}^{2+}$ leak caused by RyR2 mutations or phosphorylation. K201 eliminated SCR and attenuated AP prolongation and DOR during bradycardia. Additionally, K201 may have off-target effects which must be carefully considered. In guinea pig ventricular myocytes, K201 (1 μM) inhibited $I_{\text{Na}}, I_{\text{Ca,L}},$ and $I_{\text{Kr}}$ in addition to its effect on RyR2 (30, 31); however, these off-target effect appear to be species dependent.

In rabbit myocytes, K201 was specific for RyR2 at 1 μM: it...
Previous studies on Ca$^{2+}$ dynamics and arrhythmias. The role of Ca$^{2+}$ handling abnormalities as a trigger of cardiac arrhythmias has been extensively investigated using Ca$^{2+}$ imaging at the subcellular level, with most studies performed on isolated myocytes (8, 10, 46), Purkinje fibers (19, 28, 53), superfused myocardial trabeculae (34, 35, 37, 48), and myocyte monolayers (7, 36). Although intact perfused hearts provide significant advantages compared with isolated preparations to study arrhythmias, technical difficulties (e.g., greater susceptibility to motion artifact, scan rate, and signal-to-noise ratio) have made subcellular Ca$^{2+}$ imaging challenging in this setting. At least three groups have now used confocal microscopy to study abnormalities of Ca$^{2+}$ in various models of cardiac arrhythmias, such as ventricular hypertrophy, hypokalemia, rapid pacing, and delayed afterdepolarization after cessation of rapid pacing (1, 2, 6, 23). A common finding was that nonvoltage-triggered diastolic Ca$^{2+}$ release often produced subcellular Ca$^{2+}$ waves that appeared to play a crucial role in the genesis of arrhythmias. However, the role of abnormal Ca$^{2+}$ dynamics will be difficult to analyze in other situations, such as ischemia, repolarization delay, or bradycardia, where longer intervals of data acquisition are needed at both high and low spatial resolution. Whereas confocal imaging in the linescan mode reaches the necessary high sampling frequency, the field of view is relatively small and cannot be switched from line-scan to macroscopic imaging to track an arrhythmia throughout the intact heart. In addition, most confocal experiments were performed at room temperature because the fluo4 fluorescence diminishes above 30°C but hypothermia can have profound effects on Ca$^{2+}$ dynamics. The method we used here overcomes some of these technical problems.

Study limitations. This investigation studied the effects of acute bradycardia and may be less clinically relevant than animal models of chronic bradycardia where considerable tissue remodeling occurs (20, 58, 59). The study focused on the epicardium and did not investigate SCR from different regions of the ventricles. Although our previous studies demonstrated higher levels of ICa,L and INCX occurred at the base of the rabbit epicardium and not on the endocardium (17, 52), we cannot exclude the possibility of SCR in other regions of the heart. In pilot studies, optical maps of APs and CaT at high and low magnification from the endocardium did not find SCR before and during bradycardia. Unfortunately, optical recordings are still limited to the surface. The study suggests that bradycardia causes an extra APD prolongation at the base by eliciting SCR and INCX augmentation. Several pharmacological agents can be used to suppress SCR by RyR2 inhibition. We used K201 because of its selectivity in rabbit hearts and because alternative agents flecaainide and ranolazine also suppress SCR but have significant effects on the INa,L and ICa,L. Pilot studies tested pharmacological blockers of NCX to evaluate more directly its impact on APD prolongation during bradycardia. Unfortunately, the available NCX inhibitors are not sufficiently selective or effective at blocking NCX. Trials with SEA0400 (0.1–2 μM) to block the forward mode of NCX were inconclusive because at these concentrations, inhibition of NCX is partial, and at concentrations > 1 μM, ICa,L is progressively suppressed (11). An alternative approach of lowering external Na$^{+}$ has been successfully used to inhibit INCX in isolated myocytes but cannot be applied fast enough in perfused hearts. Likewise, caffeine can be effectively used to estimate SR Ca$^{2+}$ load in isolated myocytes but not in perfused hearts. Nevertheless, these limitations do not detract from the validity of the study which exemplifies what can be done at the intact heart level to fully appreciate heterogeneities and complexities that cannot be exposed in studies with isolated myocytes.

In summary, the study investigates acute abnormalities of myocardial Ca$^{2+}$ handling caused by bradycardia, which increases propensity to ectopic activity and promotes arrhythmia. A recent elegant study has shown the role of remodeling of Ca$^{2+}$ handling processes for arrhythmia in chronic bradycardia (58). At the subcellular level, the mechanisms of SCR clearly merit additional investigation. Clinically, it is possible that RyR2 stabilizers and NCX blockers could have a role in acute treatment of bradycardia-induced TdP, at least until pacing therapy can be instituted.

GRANTS
The study was supported in part by National Heart, Lung, and Blood Institute Grants HL-70722 and HL-093074 (to G. Salama) and a Three Rivers Affiliate of the American Heart Association Pre-doctoral Fellowship (to J. J. Kim) and Post-doctoral Fellowship (to R. Papp).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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