intracellular calcium (Cai) waves. Am J Physiol Heart Circ Physiol 297: H997–H1002, 2009. First published June 26, 2009; doi:10.1152/ajpheart.00390.2009.—Intracellular Ca2+ (Cai) waves are known to cause delayed afterdepolarizations (DADs), which have been associated with arrhythmias in cardiac disease states such as heart failure, catecholaminergic polymorphic ventricular tachycardia, and digitalis toxicity. Here we show that, in addition to DADs, Cai2+ waves also have other consequences relevant to arrhythmogenesis, including subcellular spatially discordant alternans (SDA, in which the amplitude of the local Cai2+ transient alternates out of phase in different regions of the same cell), sudden repolarization changes promoting the dispersion of refractoriness, and early afterdepolarizations (EADs). Cai2+ was imaged using a charge-coupled device-based system in fluo-4 AM-loaded isolated rabbit ventricular myocytes paced at constant or incrementally increasing rates, using either field stimulation, current clamp, or action potential (AP) clamp. Cai2+ waves were induced by Bay K 8644 (50 nM) + isoproterenol (100 nM), or low temperature. When pacing was initiated during a spontaneous Cai2+ wave, SDA occurred abruptly and persisted during pacing. Similarly, during rapid pacing, SDA typically arose suddenly from spatially concordant alternans, due to an abrupt phase reversal of the subcellular Cai2+ transient in a region of the myocyte. Cai2+ waves could be visualized interspersed with AP-triggered Cai2+ transients, producing a rich variety of subcellular Cai2+ transient patterns. With free-running APs, complex Cai2+ release patterns were associated with DADs, EADs, and sudden changes in AP duration. These findings link Cai2+ waves directly to a variety of arrhythmogenic phenomena relevant to the intact heart.

calcium transient; cardiac myocytes; discordant alternans; sarcoplasmic reticulum; ryanodine receptor

INTRACELLULAR CALCIUM (Cai) waves in the setting of sarcoplasmic reticulum (SR) Ca2+ overload cause delayed afterdepolarizations (DADs) by activating transient inward currents due to electrogenic Na+-Ca2+ exchange and/or Ca2+-activated nonselective cation channels (7, 17). DADs can lead to triggered activity, an important mechanism causing atrial and ventricular arrhythmias in a variety of cardiac diseases, including heart failure, catecholaminergic polymorphic ventricular tachycardia, and digitalis toxicity. Here we show that in addition to DADs, Cai2+ waves have other consequences relevant to arrhythmogenesis, including subcellular spatially discordant alternans (SDA), abrupt action potential (AP) repolarization changes, and early afterdepolarizations (EADs).

SDA can occur at both the cellular and tissue levels. At the tissue level, beat-to-beat alternans in AP duration (APD) creates a highly arrhythmogenic substrate favoring reentry and fibrillation when it becomes spatially discordant (4, 12, 16, 20). Since the Ca2+ transient is coupled to the AP through Ca2+-sensitive ion channels and electrogenic transporters, Cai2+ alternans, defined as the beat-to-beat alternation of the Ca2+ transient amplitude, can play an important role in the genesis of electrical APD alternans (5, 8, 14, 15). At the cellular level, Cai2+ alternans can occur in two forms. In spatially discordant alternans (SCA), the amplitudes of subcellular Cai2+ transients alternate in phase throughout all the regions of the myocyte. In SDA, however, the amplitude of Cai2+ release alternates out of phase in different regions of the same cell. Both SCA and SDA generally promote APD alternans. However, if SDA is balanced such that the Cai2+ transient in half of the myocyte alternates out of phase with the other half so that the summed whole cell Cai2+ transient is invariant, APD alternans is less evident (10). The cause of subcellular SDA of the Ca2+ transient has not been clearly determined, although several mechanisms have been postulated (18). Here we show direct evidence that local phase resetting by Cai2+ waves can cause subcellular SDA. We also show that the interaction between spontaneous Cai2+ waves, DADs, and AP-triggered SR Ca2+ release can produce a variety of complex subcellular Ca2+ dynamics, leading to sudden changes in repolarization and EADs.

MATERIALS AND METHODS

Cell isolation. Ventricular myocytes were enzymatically isolated from adult rabbit hearts. Briefly, the hearts were removed from adult New Zealand White rabbits (2 to 3 kg), anesthetized with intravenous pentobarbital sodium and perfused retrogradely in Langendorff fashion at 37°C with nominally Ca2+-free Tyrode solution containing ~1.4 mg/ml collagenase (type II; Worthington) and 0.1 mg/ml protease (type XIV, Sigma) for 25–30 min. After the enzyme solution was washed out, the hearts were removed from the perfusion apparatus and swirled in a culture dish. The Ca2+ concentration was slowly increased to 1.8 mM, and the cells were stored at room temperature and used within 8 h. The use and care of the animals in these experiments were reviewed and approved by the Chancellor’s Animal Research Committee at the University of California, Los Angeles, and the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey-New Jersey Medical School.

Patch-clamp methods. Myocytes were patch clamped using the whole cell configuration of the patch-clamp technique in the current-clamp or voltage-clamp mode. Patch pipettes (resistance, 2–4 MΩ) were filled with internal solution containing (in mM) 110 K+-aspartate, 30 KCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 5 MgATP, 5 creatine phosphate, and 0.05 cAMP (pH 7.2) with KOH. Myocytes were superfused with standard Tyrode solution containing (in mM) 136 NaCl, 5.4 KCl, 0.33 Na2PO4, 1.8 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES (pH 7.4), adjusted with NaOH. APs were elicited with 2-ms, 2–4-nA square pulses at various pacing cycle lengths (PCLs). Voltage signals were measured using an Axopatch 200B patch-clamp amplifier.
controlled by a personal computer using a Digitata 1200 acquisition board, driven by pCLAMP 8.0 software.

**Ca**\(^{2+}\) measurement. Myocytes were loaded with the Ca**\(^{2+}\)** indicator fluo-4 by incubating them for \(\sim30\) min in bath solution containing 4 \(\mu\)M fluo-4 AM (Molecular Probes) and 0.016% (wt/wt) pluronic (Molecular Probes), after which the cells were washed and placed in a heated chamber on an inverted microscope. Ca**\(^{2+}\)** fluorescence was recorded using an Andor Ixon charge-coupled device camera (Andor Technology) operating at \(\sim100\) frames/s with a spatial resolution of 512 \(\times\) 180 pixels. Fluorescence intensity was recorded as the ratio of fluorescence over the basal diastolic fluorescence.

**Cell stimulation.** Myocytes were either field stimulated, current clamped to record free-running APs simultaneously, or voltage clamped with a fixed AP waveform (AP clamp). Bay K 8644 (BayK) and/or isoproterenol (Iso) were applied to initiate subcellular SCA during paced, either at a constant PCL or during incremental rapid pacing, in which PCL progressively decreased from 500 to 150 ms in \(\sim20\)-ms decrements every 12 beats.

Most experiments were carried out at \(\sim35^\circ\)C, and some at room temperature (22–24°C).

**RESULTS**

With the use of either field stimulation or whole cell current clamp to record the AP simultaneously, none of 21 rabbit ventricular myocytes exhibited spontaneous Ca**\(^{2+}\)** waves under control conditions. When paced at progressively shorter PCL until loss of 1:1 capture, 16 of the 21 myocytes developed SCA of the Ca**\(^{2+}\)** transient, but only 2 progressed to SDA. With the use of an AP clamp to prevent concomitant APD alternans, 15 of 17 developed SCA, but none developed SDA.

In 10 myocytes superfused with the L-type Ca**\(^{2+}\)** current agonist BayK (50 nM) and the \(\beta\)-adrenergic agonist Iso (100 nM) to promote Ca**\(^{2+}\)** overload, 4 exhibited spontaneous SR Ca**\(^{2+}\)** waves. During rapid incremental pacing, SDA occurred in 8 of 10 myocytes with free-running APs and in 5 out of 7 myocytes paced with a fixed AP waveform (AP clamp). Thus BayK and Iso promoted both spontaneous Ca**\(^{2+}\)** waves and SDA, and SDA did not require the AP to be free running (18).

The ability of a spontaneous Ca**\(^{2+}\)** wave to induce SDA suddenly by a phase-resetting mechanism is illustrated in Fig. 1A. A spontaneous Ca**\(^{2+}\)** wave originating near the middle of the myocyte was propagating toward the two ends just as pacing with an AP clamp at a PCL of 400 ms was initiated. During the first AP clamp, Ca**\(^{2+}\)** at the middle of the myocyte (where the wave originated) had already recovered to \(\sim20\)% of the baseline diastolic level, whereas at the ends of the myocyte, Ca**\(^{2+}\)** was still high (\(\sim60\)% above the baseline diastolic level). This resulted in a large Ca**\(^{2+}\)** release from the middle region where Ca**\(^{2+}\)** had recovered but only small Ca**\(^{2+}\)** releases from the ends of the cell where the SR Ca**\(^{2+}\)** release machinery was still refractory. The pattern reversed on the next AP clamp (Fig. 1A, 2nd arrow), and so forth, resulting in a maintained SDA. The same phenomena as shown in Fig. 1 were observed in three other cells. This finding demonstrates that a properly timed Ca**\(^{2+}\)** wave can reset the phase of Ca**\(^{2+}\)** alternans regionally to initiate SDA, whether SCA is present or not.

In myocytes developing SDA during pacing, the transition from SCA to SDA typically occurred suddenly. Figure 2 illustrates an example. In this myocyte, no alternans was present before BayK and Iso during pacing at a PCL of 500 ms. The addition of BayK and Iso markedly potentiated the Ca**\(^{2+}\)** transient, which then began to alternate in a spatially concor-
leased by the wave was still refractory, it responded to the paced beat with less Ca\textsuperscript{2+} release relative to the ends of the myocyte. This allowed a longer time for the refilling of SR Ca\textsuperscript{2+} before repolarization was complete. The localized spontaneous SR Ca\textsuperscript{2+} release reduced the repolarization reserve sufficiently (by activating electrogenic Na\textsuperscript+-Ca\textsuperscript{2+} exchange and/or other Ca\textsuperscript{2+}-sensitive inward currents) to cause an EAD. The reactivation of the L-type Ca\textsuperscript{2+} current during the EAD led to an additional triggered SR Ca\textsuperscript{2+} release in nearby regions (dashed arrows). The third paced beat induced a similar AP as the first paced beat and was also followed by a Ca\textsuperscript{2+} wave causing a DAD. This sequence directly illustrates that during pacing, interspersed Ca\textsuperscript{2+} waves can produce a variety of effects via their feedback on membrane potential, including marked sudden changes in APD and the generation of both EADs and DADs. In Fig. 3C, it is notable that during the fourth paced beat, the Ca\textsuperscript{2+} release from the ends of the myocyte (into which the preceding Ca\textsuperscript{2+} wave had failed to propagate) is not as large as we might have expected, given the substantial recovery period. Although we do not have a ready explanation, the decreased SR Ca\textsuperscript{2+} load due to the increased Ca\textsuperscript{2+} extrusion by the Na\textsuperscript{+-}Ca\textsuperscript{2+} exchanger current (transient inward current causing the DAD) might be one possible cause.

The development of SDA was usually preceded by SCA, as illustrated in Fig. 2. However, in one myocyte, we observed the reverse sequence (Fig. 4). The cell was paced with a free-running AP under control conditions. At a PCL of 400 ms, neither APD nor Ca\textsuperscript{2+} alternans was present (Fig. 4A), but when PCL decreased to 300 ms, SDA developed (Fig. 4B).

**Fig. 2.** Sudden conversion of spatially concordant alternans (SCA) to SDA during rapid pacing. A: line scan of Ca\textsuperscript{2+} fluorescence along the midline of a fluo-4-loaded rabbit ventricular myocyte, field stimulated at a PCL of 500 ms before (left) and during (right) exposure to 50 nM Bay K 8644 and 100 nM isoproterenol (BayK + Iso). B: traces of normalized Ca\textsuperscript{2+} (F/F0) from site a in the middle of the cell (red trace) and site b at the bottom end of the cell (blue trace). Initially, the Ca\textsuperscript{2+} transients at both sites alternate concordantly in a L-S pattern. At the arrow, two small Ca\textsuperscript{2+} transients occur in a row at site b, suddenly reversing the phase of alternans relative to site a. The SDA amplitude grows larger during paced subsequent beats, as seen in both the traces and line scan.

**Fig. 3.** Interaction between spontaneous Ca\textsuperscript{2+} waves and AP-evoked Ca\textsuperscript{2+} transients in the presence of 50 nM BayK and 100 nM Iso. A: typical example of spontaneous Ca\textsuperscript{2+} waves after sudden cessation of pacing in a current-clamped myocyte stimulated at a pacing cycle length (PCL) of 400 ms. Each Ca\textsuperscript{2+} wave causes a delayed afterdepolarization (DAD), which become progressively smaller. B: complex patterns of spontaneous Ca\textsuperscript{2+} waves interspersed with AP-evoked Ca\textsuperscript{2+} transients in a myocyte paced using an AP clamp at a PCL of 250 ms. B, right: an expanded segment, corresponding to the thick horizontal bar at left. Solid lines indicate the paced excitations; dashed arrows show Ca\textsuperscript{2+} waves intermixed. See also supplemental movie 1 (note: supplemental material is posted with the online version of this article). C: spontaneous Ca\textsuperscript{2+} waves interspersed with paced beats in a current-clamped myocyte stimulated at PCL of 1 s. Regional Ca\textsuperscript{2+} transients are heterogeneous, and the corresponding APs are associated with both early afterdepolarizations (EADs) and DADs. C, right: an expanded segment corresponding to the thick horizontal bar at left, labeling as described in B. See also supplemental movie 2.
Although the most likely explanation for this sudden phase reversal is a spontaneous Ca\(^{2+}\) wave, as in Fig. 1, we could not conclusively identify a Ca\(^{2+}\) wave in the fluorescence images at this relatively short PCL. Note that despite the marked SDA of the Ca\(^{2+}\) transient, the APD alternation was minimal, since the summed Ca\(^{2+}\) transient over the whole cell was relatively constant (Fig. 4B). When PCL was subsequently decreased to 210 and then 200 ms, however, the phase of the Ca\(^{2+}\) transient at the end of the cell suddenly reversed (i.e., 2 small Ca\(^{2+}\) transients in a row, indicated by arrows), so that the alternans phase in this region was now in phase with the rest of the cell, thereby converting SDA to SCA (Fig. 4C). Note that the amplitude of the whole cell Ca\(^{2+}\) transient alternans was much less during SDA than after the conversion to SCA. As a result, APD alternans amplitude also suddenly increased, illustrating how the regional phase resetting of the subcellular Ca\(^{2+}\) transient can abruptly change APD over the course of a single beat. If the sudden change in APD occurs differentially at various locations within the heart, this would abruptly amplify the dispersion of refractoriness over the course of a single beat.

In addition to treatment by Iso and BayK, we found that a lower temperature (22–24°C) also promoted SDA in six/seven myocytes (86%). Spontaneous Ca\(^{2+}\) waves occurred following the cessation of rapid pacing in all six cases (data not shown).

### DISCUSSION

Ca\(^{2+}\) waves are accepted as a classic mechanism underlying DADs and DAD-induced triggered activity. In the present study, we show that Ca\(^{2+}\) waves also have other effects that can influence arrhythmogenesis by promoting subcellular SDA, sudden repolarization changes, and EADs. When extrapolated to the tissue level, these actions may promote both DADs and EADs as triggers, while at the same time enhancing substrate vulnerability by generating APD dispersion, thereby predisposing the heart to reentry and fibrillation. Based on these multiple interactive effects, we speculate that premature ventricular complexes (PVCs) arising from DADs in diseased hearts predisposed to Ca\(^{2+}\) waves may be intrinsically more likely to initiate lethal ventricular arrhythmias than PVCs due to other mechanisms, which are generally benign in the absence of associated heart disease (13). This may contribute to the higher risk of sudden cardiac death in clinical settings such as heart failure, catecholaminergic polymorphic ventricular tachycardia, and digitalis toxicity in which excitation-contraction-coupling remodeling, genetic defects, or drugs predispose myocytes to Ca\(^{2+}\) waves and DADs.

**Ca\(^{2+}\) waves and arrhythmias in tissue.** Our study does not directly address how, by altering AP characteristics at the cellular level, Ca\(^{2+}\) waves scale to the tissue level to cause arrhythmias. However, a recent two-photon confocal imaging study in intact arterially perfused rat ventricle (7) demonstrated that rapid pacing naturally synchronized Ca\(^{2+}\) waves in a sufficient mass of adjacent myocytes to cause frank DADs in the tissue. In contrast, sporadic Ca\(^{2+}\) waves in individual myocytes at slow heart rates had no effect on local membrane potential due to the source-sink mismatch. Extrapolating from these results, we can sketch possible scenarios illustrating the arrhythmogenic consequence of Ca\(^{2+}\) waves in tissue. For example, the scenario in Fig. 1 may be relevant to rapid heart beating followed by a pause long enough to allow the overcharged SR to spontaneously release Ca\(^{2+}\). If a sinus or escape beat occurs while Ca\(^{2+}\) waves are still propagating, it could induce SDA in all of the cells in which the Ca\(^{2+}\) wave had not yet propagated the full length of the cell and thereby influence regional APD dispersion. As another possible example is if we suppose that rapid pacing has induced spatially concordant APD and Ca\(^{2+}\) transient alternans in tissue. If the myocytes in a region of the tissue synchronously develop Ca\(^{2+}\) waves (7), resulting in subcellular SDA, the amplitude and possibly even the phase of APD alternans in that region may change relative to the surrounding unperturbed tissue, increasing spatial APD dispersion. If documented experimentally in tissue, these scenarios would represent novel mechanisms for the formation of arrhythmogenic spatially discordant APD alternans at the tissue level. Similarly, regionally synchronous Ca\(^{2+}\) waves that cause abrupt regional changes in APD or generate EADs may likewise exacerbate APD dispersion and/or EAD-related triggered activity. On the other hand, the subcellular SDA also has the potential to be antiarrhythmic by reducing the amplitude and spatial heterogeneity of APD alternans in tissue if it occurs globally rather than regionally.

**Mechanism of subcellular SDA.** Although a steep APD restitution slope, preexisting tissue heterogeneity, and intrinsic dynamics of Ca\(^{2+}\) cycling can all promote APD alternans,
recent evidence favors the latter mechanism as causing the initial instability as heart rate increases (9, 18). Combined experimental and theoretical studies have helped to illuminate the mechanisms of “primary” Ca$^{2+}$ alternans (i.e., Ca$^{2+}$ alternans that is independent of APD alternans), but most modeling studies have focused on the whole cell Ca$^{2+}$ transient behavior rather than subcellular patterns of Ca$^{2+}$ release.

Subcellular SDA has been observed in single cells (3, 6, 11) and intact tissue (1, 2), and possible theoretical mechanisms have been proposed (2, 18). However, direct experimental evidence for any of the theoretical mechanisms is incomplete. The present study presents the direct experimental evidence for a novel mechanism, i.e., the phase resetting of SR Ca$^{2+}$ by Ca$^{2+}$ wave. Conceptually, the mechanism producing spatially discordant Ca$^{2+}$ alternans at the subcellular scale is analogous to the phase-resetting mechanism by which a PVC can induce spatially discordant APD alternans at the tissue scale (20). In the latter case, the different activation sequence of the PVC results in the next paced beat experiencing a tissue gradient in diastolic intervals, which generates a corresponding APD gradient. In the former case, the partially propagated Ca$^{2+}$ wave generates a gradient in SR refractoriness when the next paced AP occurs. In the region of the myocyte through which the Ca$^{2+}$ wave has already passed, the affected SR is empty and partially refractory, minimizing Ca$^{2+}$ release during the AP. In contrast, the region into which the Ca$^{2+}$ wave has not penetrated releases a normal amount of SR Ca$^{2+}$ during the AP, resulting in a spatially nonuniform Ca$^{2+}$ transient. On the next AP, the refractory region will have recovered both SR Ca$^{2+}$ content and excitability, producing a large release, and so forth, perpetuating SDA. Figure 1 directly demonstrated this phenomenon. The refractoriness of local Ca$^{2+}$ release units has been experimentally documented by Sobie et al. (19), who measured a time constant of ~90 ms in rat ventricular myocytes. The sudden phase reversals of the regional Ca$^{2+}$ transient, which we observed, are fully consistent with this mechanism as the cause of the abrupt onset of SDA (Fig. 2), as well as the sudden conversion of SDA to SCA (Fig. 4). Unfortunately, the limitations in the spatial and temporal resolution of our imaging system precluded the direct visualization of the putative culprit spontaneous Ca$^{2+}$ waves causing these phase reversals in all cases. However, the examples in Figs. 3, A–C, clearly show that when myocytes were sufficiently Ca$^{2+}$ overloaded, spontaneous Ca$^{2+}$ waves became interspersed with the AP-triggered Ca$^{2+}$ transients, producing not only SDA but also more complex subcellular patterns of Ca$^{2+}$ release (Fig. 3C). Confocal imaging may have improved our ability to detect spontaneous Ca$^{2+}$ waves, causing regional phase reversals. However, the imaging plane would have to be fortuitously placed at just the right depth. We preferred nonconfocal imaging in this initial study to visualize the Ca$^{2+}$ transient through the full thickness of the myocyte, to maximize our chances of detecting Ca$^{2+}$ waves originating from any depth. Given an average myocyte thickness of ~10 μm, this sacrifices spatial resolution in the z-axis by approximately fivefold compared with that in confocal imaging.

Alternative mechanisms of subcellular SDA. In addition to phase resetting by Ca$^{2+}$ waves, other mechanisms of subcellular SDA have also been proposed. Shiferaw et al. (18) predicted theoretically that the nonlinear interactions between membrane voltage and Ca$^{2+}$ cycling could lead to complex patterns of subcellular SDA, under conditions in which the coupling between Ca$^{2+}$ and APD is negative (i.e., a large Ca$^{2+}$ transient shortens APD by enhancing the Ca$^{2+}$-induced inactivation of the L-type Ca$^{2+}$ current). Experimentally, however, we typically observed a positive Ca$^{2+}$-APD coupling in rabbit ventricular myocytes (e.g., Fig. 4) yet could still induce SDA. Also, SDA, due to this dynamic mechanism, would be expected to develop gradually, not suddenly, during a single beat, as observed in Figs. 1 and 4. Therefore, this dynamic mechanism is unlikely to account for a subcellular SDA in rabbit myocytes under the specific conditions of our study. However, our findings do not exclude the potential importance of this mechanism under different experimental conditions.

Another possibility that might explain SDA is the preexisting heterogeneity in the Ca$^{2+}$ cycling machinery, i.e., the regional differences in the density of L-type Ca$^{2+}$ channels, SR Ca$^{2+}$ release units, SR Ca$^{2+}$ refilling kinetics, etc., causing different thresholds for the onset of alternans in different regions of the cell (13). In this case, one might expect SDA to arise de novo, without preceding SCA. However, de novo SDA was observed in only 2 of 38 myocytes, and in one of these myocytes, SDA subsequently abruptly converted to SCA (Fig. 4). It is not intuitively obvious how preexisting subcellular regional heterogeneity in the Ca$^{2+}$ cycling machinery’s threshold for alternans could account for a sudden transition from SDA to SCA.

Thus we do not discount the importance of either preexisting of subcellular heterogeneity in Ca$^{2+}$ cycling components (3, 6, 11) or purely dynamical mechanisms (18) in causing subcellular SDA. Rather, we provide direct experimental evidence for an additional, novel mechanism that we believe is the cause of SDA under our particular experimental condition.

Summary. In summary, our observations demonstrate that under control conditions at near physiological temperature, spontaneous Ca$^{2+}$ waves and pacing-induced SDA were both infrequent in rabbit ventricular myocytes. Under conditions (pharmacological, ionic, or lower temperature) in which myocytes became susceptible to Ca$^{2+}$ overload during rapid pacing, spontaneous Ca$^{2+}$ waves and AP-triggered Ca$^{2+}$ transients produced SDA and even more complex subcellular Ca$^{2+}$ transient patterns in a high percentage of myocytes. Moreover, when the AP was free running, these spatially complex Ca$^{2+}$ release patterns due to Ca$^{2+}$ waves caused DADs and EADs, as well as marked sudden changes in APD (Fig. 4) as a result of the coupling between Ca$^{2+}$ release and voltage (Fig. 3C). Complex subcellular Ca$^{2+}$ cycling dynamics may therefore provide a direct link to the triggers and dispersion of the electrophysiological properties that promote arrhythmias in the intact heart.

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