Refractoriness of sarcoplasmic reticulum Ca\textsuperscript{2+} release determines Ca\textsuperscript{2+} alternans in atrial myocytes

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Cardiac alternans is a recognized risk factor for cardiac arrhythmia and sudden cardiac death. At the cellular level, Ca\textsuperscript{2+} alternans appears as cytosolic Ca\textsuperscript{2+} transients of alternating amplitude at regular beating frequency. Cardiac alternans is a multifactorial process but has been linked to disturbances in intracellular Ca\textsuperscript{2+} regulation. In atrial myocytes, we tested the role of voltage-gated Ca\textsuperscript{2+} current, sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} load, and restitution properties of SR Ca\textsuperscript{2+} release for the occurrence of pacing-induced Ca\textsuperscript{2+} alternans. Voltage-clamp experiments revealed that peak Ca\textsuperscript{2+} current was not affected during alternans, and alternans of end-diastolic SR Ca\textsuperscript{2+} load, evaluated by application of caffeine or measured directly with an intra-SR fluorescent Ca\textsuperscript{2+} indicator (fluo-5N), were not a requirement for cytosolic Ca\textsuperscript{2+} alternans. Restitution properties and kinetics of refractoriness of Ca\textsuperscript{2+} release after activation during alternans were evaluated by four different approaches: measurements of $I$ the delay (latency) of occurrence of spontaneous global Ca\textsuperscript{2+} releases and 2) Ca\textsuperscript{2+} spark frequency, both during rest after a large and small alternans Ca\textsuperscript{2+} transient; 3) the magnitude of premature action potential-induced Ca\textsuperscript{2+} transients after a large and small beat; and 4) the efficacy of a photolytically induced Ca\textsuperscript{2+} signal (Ca\textsuperscript{2+} uncaging from DM-nitrophen) to trigger additional Ca\textsuperscript{2+} release during alternans. The results showed that the latency of global spontaneous Ca\textsuperscript{2+} release was prolonged and Ca\textsuperscript{2+} spark frequency was decreased after the large Ca\textsuperscript{2+} transient during alternans. Furthermore, the restitution curve of the Ca\textsuperscript{2+} transient elicited by premature action potentials or by photolysis-induced Ca\textsuperscript{2+} release from the SR lagged behind after a large-amplitude transient during alternans compared with the small-amplitude transient. The data demonstrate that beat-to-beat alternation of the time-dependent restitution properties and refractory kinetics of the SR Ca\textsuperscript{2+} release mechanism represents a key mechanism underlying cardiac alternans.

calcium alternans; ryanodine receptor; refractoriness; sarcoplasmic reticulum calcium release

Nonetheless, it has become increasingly clear that alternans is ultimately linked to disturbances in myocardial Ca\textsuperscript{2+} homeostasis and impaired intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) regulation (for reviews, see Refs. 6, 11, 17, 19, 21, 22, 37, 41, and 48). Based on the theory of cardiac Ca\textsuperscript{2+} cycling, computational studies, and experimental data (for reviews and references, see Refs. 63 and 64), two parameters have emerged as critically relevant to the beat-to-beat regulation of [Ca\textsuperscript{2+}]\textsubscript{i} and the generation of alternans: fractional Ca\textsuperscript{2+} release and the efficiency of cytosolic Ca\textsuperscript{2+} sequestration. Fractional release of Ca\textsuperscript{2+} refers to the nonlinear relationship between the Ca\textsuperscript{2+} content of the sarcoplasmic reticulum (SR) and the amount of Ca\textsuperscript{2+} (or fraction of SR Ca\textsuperscript{2+} content) released by Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) with each cardiac cycle, where at a higher SR Ca\textsuperscript{2+} content a larger fraction is liberated upon activation of CICR (3). Ca\textsuperscript{2+} sequestration is a phenomenological parameter that refers to the net efficiency of clearing the cytosolic compartment of Ca\textsuperscript{2+} and includes Ca\textsuperscript{2+} reuptake into the SR via sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA), extrusion via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) and plasmalemmal Ca\textsuperscript{2+}-ATPase, cytosolic buffering, mitochondrial uptake, and diastolic SR Ca\textsuperscript{2+} leak via ryanodine receptor (RyR)-mediated SR Ca\textsuperscript{2+}-release channels or other pathways; see Ref. 69). The nonlinear relationship between Ca\textsuperscript{2+} sequestration and fractional release determines the vulnerability to alternans (see Fig. 6 in Ref. 63). The relationship predicts that, in general, factors increasing Ca\textsuperscript{2+} load and fractional release promote alternans and factors increasing Ca\textsuperscript{2+} sequestration protect against alternans. Thus, under low Ca\textsuperscript{2+} sequestration conditions (e.g., because of reduced SERCA activity or enhanced diastolic SR Ca\textsuperscript{2+} leak) alternans can occur at relatively modest SR loads and small fractional releases, whereas at high sequestration rates higher Ca\textsuperscript{2+} loads and fractional release are required to induce alternans. The boundary between stability and instability (alternans) of Ca\textsuperscript{2+} cycling is not linear. The boundary is shallow in the low sequestration range and becomes steep at high sequestration rates. That is, at low sequestration rates the transition from stability to instability is dominated by changes in fractional release, whereas at higher sequestration rates small changes in Ca\textsuperscript{2+} sequestration become critical for the occurrence of alternans.

The beat-to-beat dynamics of both Ca\textsuperscript{2+} sequestration and fractional release are critically dependent on the restitution properties and refractory kinetics of the SR Ca\textsuperscript{2+}-release mechanism. The amount of Ca\textsuperscript{2+} released during a given heart beat, and thus the magnitude of the Ca\textsuperscript{2+} transient, is determined by the recovery of the trigger of CICR, SR Ca\textsuperscript{2+} load, and the release mechanism itself (RyRs and associated regulatory proteins) of the preceding beat. APD restitution (including the recovery of L-type Ca\textsuperscript{2+} channels from voltage- and...

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Ca\textsuperscript{2+}-dependent inactivation) has been recognized as causative and/or contributing factors to electromechanical and Ca\textsuperscript{2+} alternans and may play a role, particularly at high heart rates (for reviews, see Refs. 63 and 64). The role of reuptake of Ca\textsuperscript{2+} into the SR and reestablishing Ca\textsuperscript{2+} load have been the subject of numerous investigations (12, 30, 68) together with the controversial question of whether cardiac alternans requires beat-to-beat alternations in SR Ca\textsuperscript{2+} content and end-diastolic SR filling (15) or not (28). While it has been suggested that instability in the beat-to-beat feedback control of SR content leads to Ca\textsuperscript{2+} alternans (18), direct dynamic measurements of Ca\textsuperscript{2+} concentration in the SR ([Ca\textsuperscript{2+}]\textsubscript{SR}) revealed that alternans can readily occur without significant diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} fluctuations (44). These results raise the intriguing possibility that factors other than SR Ca\textsuperscript{2+} load, such as the kinetics of restitution of SR Ca\textsuperscript{2+} release (or time dependence of the refractoriness of the Ca\textsuperscript{2+}-release mechanism) centrally underlie pacing-induced cardiac alternans. Indeed, recent findings have provided evidence that the recovery of CICR or refractoriness of RyR-mediated Ca\textsuperscript{2+} release may contribute to the instabilities of cardiac Ca\textsuperscript{2+} release and vulnerability to arrhythmias (56). Experimental studies on the recovery of Ca\textsuperscript{2+} transients as well as Ca\textsuperscript{2+} sparks (i.e., Ca\textsuperscript{2+} release at the local level of individual SR Ca\textsuperscript{2+}-release units) (7, 8, 47, 52, 55, 57) have raised the possibility that the recovery of RyR-dependent Ca\textsuperscript{2+} release may occur on a time scale that overlaps with the stimulation frequencies at which Ca\textsuperscript{2+} alternans occurs. The time-dependent recovery of SR Ca\textsuperscript{2+} release may become a critical factor for the occurrence of Ca\textsuperscript{2+} alternans when recovery is slowed, a mechanism we have proposed to contribute to alternans during inhibition of glycolysis (28).

Therefore, the goal of the present investigation was to test the hypothesis that beat-to-beat alternations in the restitution kinetics of SR Ca\textsuperscript{2+} release cause Ca\textsuperscript{2+} alternans in atrial tissue. The focus was placed on atrial myocytes for several reasons: 1) atrial arrhythmias (particularly atrial fibrillation) are the most common and most prevalent form of cardiac arrhythmia (e.g., Ref. 43) and have been linked directly to alternans (26, 42); 2) at the cellular level, due to the lack of a t-tubular membrane system and the special features of Ca\textsuperscript{2+} release during excitation-contraction coupling (27, 33, 39, 40, 53, 66), atrial myocytes are particularly susceptible to pacing and metabolically induced Ca\textsuperscript{2+} alternans (28, 34); and 3) Ca\textsuperscript{2+} alternans can be subcellularly inhomogeneous (1, 2, 15, 24), which is again particularly pronounced in atrial tissue (28, 32). We have demonstrated subcellular transverse and longitudinal gradients of the degree of Ca\textsuperscript{2+} alternans and subcellular regions alternating out of phase. These pronounced and complex subcellular inhomogeneities and gradients provide a proarrhythmic substrate that renders atrial myocytes particularly vulnerable to spontaneous SR Ca\textsuperscript{2+} release and susceptible to propagating arrhythmogenic diastolic Ca\textsuperscript{2+} waves. The present study provides the first direct evidence that in atrial myocytes, beat-to-beat alternation of the restitution kinetics of SR Ca\textsuperscript{2+} release represents a key causative mechanism for the occurrence of electromechanical and Ca\textsuperscript{2+} alternans.

Part of this work has been previously presented in abstract form (54).

**METHODS**

**Solutions and chemicals.** All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Tyrode solution contained (in mM) 135 NaCl, 4 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 d-glucose, and 10 HEPES (pH 7.4 with NaOH).

**Myocyte isolation.** Atrial myocytes were isolated from New Zealand White rabbits (2.5 kg, Myrtle’s Rabbity, Thompsons Station, TN). Rabbits were anesthetized with pentobarbital sodium (50 mg/kg), and hearts were excised and mounted on a Langendorff apparatus. Hearts were retrogradely perfused with nominally Ca\textsuperscript{2+}-free Tyrode solution containing heparin (2 U/ml) for 5 min, followed by minimal essential medium Eagle (MEM) solution containing 20 μM Ca\textsuperscript{2+} and 45 μg/ml Liberase Blendzyme TH (Roche Applied Science, Indianapolis, IN) for 20 min at 37°C. MEM solution contained (in mM) 23.8 NaHCO\textsubscript{3}, 2 Na-pyruvate, 10 Na-HEPES, 10 HEPES, 0.02 CaCl\textsubscript{2}, and 8 tauarine with 40 U/ml insulin and 50,000 U/ml penicillin-streptomycin (pH 7.35 with NaOH). Digested tissue was minced and dispersed in MEM solution containing 30 mg/ml BSA and 10 μM Ca\textsuperscript{2+}. Finally, isolated atrial cells were kept in MEM solution with 50 μM CaCl\textsubscript{2} and were used within 1–6 h after isolation. All experiments were performed at room temperature (22–24°C). Animal protocols were approved by the Institutional Animal Care and Use Committee.

**Confocal microscopy and [Ca\textsuperscript{2+}] measurements.** Confocal microscopy was used for the measurements of intra-SR Ca\textsuperscript{2+}, cytosolic Ca\textsuperscript{2+} transients, Ca\textsuperscript{2+} sparks, and Ca\textsuperscript{2+} waves. To directly monitor [Ca\textsuperscript{2+}]\textsubscript{SR}, the SR was loaded with the low-affinity Ca\textsuperscript{2+} indicator fluo-5N (Molecular Probes-Invitrogen, Carlsbad, CA) by incubation of atrial myocytes with 10 μM of membrane-permeable fluo-5N AM together with 0.25% Pluronic F-127 in nominally Ca\textsuperscript{2+}-free Tyrode solution for 2.5 h, followed by a 30-min wash (all at 37°C). For [Ca\textsuperscript{2+}]\textsubscript{i} measurements, intact atrial myocytes were incubated with 10 μM fluo-4 AM (Molecular Probes-Invitrogen) for 10 min, followed by a 15-min wash. Cells were placed on laminin-coated glass coverslips. [Ca\textsuperscript{2+}]\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{SR} were measured with fluorescence laser scanning confocal microscopy (Radiance 2000 MP, Bio-Rad). Fluo-4 and fluo-5N were excited using the 488-nm line of an argon ion laser, and emission signals were acquired at >500 nm. All line-scan images (3 ms/line, 0.1-μm pixel size) were recorded from a central focal plane, with the scan line positioned along the transverse axis of the cell, avoiding regions of the nucleus. This allowed recording of Ca\textsuperscript{2+} release simultaneously from the junctional SR (j-SR) in the cell periphery and the nonjunctional SR (nj-SR) in the cell center (27). Background-subtracted fluorescence emission signal (F) were normalized to baseline fluorescence (F\textsubscript{0}) and changes of [Ca\textsuperscript{2+}]\textsubscript{i}, are presented as changes of F/F\textsubscript{0} or ΔF/F\textsubscript{0} (where ΔF = F – F\textsubscript{0}). SR Ca\textsuperscript{2+} load was assessed from the cytosolic fluo-4 signal in response to the rapid application of 10 mM caffeine. SR Ca\textsuperscript{2+} loads were quantified as the amplitude (ΔF/F\textsubscript{0}) of caffeine-induced cytosolic Ca\textsuperscript{2+} transient. Changes in [Ca\textsuperscript{2+}]\textsubscript{SR} measured with fluo-5N are expressed as [Ca\textsuperscript{2+}]\textsubscript{SR} = (F – F\textsubscript{min})/F\textsubscript{0} – F\textsubscript{min}, where F\textsubscript{0} is the baseline diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} and F\textsubscript{min} is the intra-SR fluorescence in the absence of Ca\textsuperscript{2+} obtained after complete emptying of SR Ca\textsuperscript{2+} with 10 mM caffeine.

**Electrophysiology and photolysis of caged Ca\textsuperscript{2+}.** Experiments involving photolysis of caged Ca\textsuperscript{2+} and simultaneous [Ca\textsuperscript{2+}]\textsubscript{i} and Ca\textsuperscript{2+} current (I\textsubscript{Ca}) measurements were performed on voltage-clamped atrial myocytes. The patch pipette solution contained (in mM) 120 l-aspartate, 120 CsOH, 20 TEA-Cl, 20 HEPES, 1 l-glutathione (reduced), 1 DM-nitrophen (EMD Chemicals, Philadelphia, PA), 0.8 K-ATP, 0.7 CaCl\textsubscript{2}, and 0.05 fluo-4 pentapotassium salt (Molecular Probes-Invitrogen) (pH 7.2 with CsOH). Free [Ca\textsuperscript{2+}]\textsubscript{i} of the pipette solution was ~150 nM. Due to competition of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} for binding to DM-nitrophen (20), Mg\textsuperscript{2+} was omitted from the pipette solution to achieve optimal loading of the cage with Ca\textsuperscript{2+}. In in vitro control experiments, we determined that under our experimental conditions at [Ca\textsuperscript{2+}] ≥ 300 nM, the uncaging Ca\textsuperscript{2+} signal was
constant and became independent of [Ca\(^{2+}\)] (data not shown). Low-Mg\(^{2+}\) conditions potentially affect RyR-dependent Ca\(^{2+}\) release due to decreased Mg\(^{2+}\) inhibition of the channel (e.g., Ref. 25). In our experiment, however, the pipette solution composition was constant and not expected to affect the comparison of Ca\(^{2+}\) signaling between the large- and small-amplitude alternans Ca\(^{2+}\) transient.

\(I_{\text{Ca}}\) was recorded with the patch-clamp technique in the whole cell configuration using an Axopatch 200a amplifier (Molecular Devices, Sunnyvale, CA). Atrial myocytes were voltage clamped and held at 

\[-40\text{ mV}. I_{\text{Ca}}\] was activated with 100-ms depolarization steps to \[+20\text{ mV}].\n
Simultaneous \([Ca^{2+}]\) measurements were obtained with a Nipkow dual-disk-type confocal laser scanning unit (CU10, Yokogawa Electric, Tokyo, Japan) attached to the side port of a Nikon Diaphot 300 inverted microscope equipped with a ×100 ultraviolet (UV)-transmitting oil objective lens (CF Fluor, numerical aperture: 1.3, Nikon).

Fluo-4 was excited with the 488-nm line of an argon ion laser connected fiber optically to the confocal unit. \([Ca^{2+}]\) signals were collected at \[+515\text{ nm}\] from single atrial myocytes using a photomultiplier tube. Photolysis of caged Ca\(^{2+}\) (DM-nitrophen) was achieved with single 355-nm light pulses (3–5 ms) from a Nd:YAG laser (Continuum MiniPulse II, Santa Clara, CA) connected via a single solid fused silica fiber to the epifluorescence port of the microscope. Synchronization of flash photolysis, \(I_{\text{Ca}}\), and \([Ca^{2+}]\), measurements and data recording and digitization were achieved using pCLAMP 10.2 software and the Axon Digidata 1440A interface (Molecular Devices).

Ca\(^{2+}\) alternans. \([Ca^{2+}]\) alternans was induced by incrementally increasing the pacing frequency until stable Ca\(^{2+}\) alternans was observed. Global Ca\(^{2+}\) transients were elicited by electrical stimulation via depolarization pulses in voltage-clamped myocytes or by electrical field stimulation of intact myocytes using a pair of platinum electrodes (voltage set at \[-50\%\] above the threshold for contraction). The frequency at which stable Ca\(^{2+}\) alternans was observed varied from cell to cell and ranged from \[>1\] to \[2.5\text{ Hz}\]. The degree of Ca\(^{2+}\) alternans was quantified as the alternans ratio (AR). The AR was defined as \[1 - \text{S/L}\], where S/L is the ratio of the small-amplitude Ca\(^{2+}\) transient (S) to the large-amplitude Ca\(^{2+}\) transient (L) during a pair of alternating Ca\(^{2+}\) transients (32, 67).

Data analysis and statistics. \([Ca^{2+}]\) spark frequency [quantified as the number of sparks per second and 100 \(\mu\text{m}\) of scanned distance (sparks s\(^{-1}\), (100 \(\mu\text{m}\)) \(^{-1}\)]) was calculated using the SparkMaster algorithm (45). Results are reported as means ± SE of n cells. Statistical significance was evaluated using Student’s t-test or Wilcoxon signed-rank test.

RESULTS

\(I_{\text{Ca}}\) and SR Ca\(^{2+}\) load during pacing-induced Ca\(^{2+}\) alternans. In an initial set of experiments, we tested the hypothesis of whether alternations in \(I_{\text{Ca}}\) or diastolic SR Ca\(^{2+}\) load are required for cytosolic Ca\(^{2+}\) alternans in atrial myocytes. Figure 1A shows simultaneous Ca\(^{2+}\) transients (a) and \(I_{\text{Ca}}\) (b) recordings from a voltage-clamped atrial myocyte. Ca\(^{2+}\) transients were evoked by membrane depolarization to \[+20\text{ mV}\] from a holding potential of \[-40\text{ mV}\]. In the example shown, stable Ca\(^{2+}\) alternans with an AR of 0.36 was observed at a stimulation frequency of 1 Hz. Peak \(I_{\text{Ca}}\) (Fig. 1A,c) was essentially identical during the large (\[-0.551\text{ nA}\]) and small (\[-0.559\text{ nA}\]) Ca\(^{2+}\) transients. On average, the ratio of \(I_{\text{Ca}}\) peak amplitude during the large transient divided by the small transient was 1.008 ± 0.011 (\(n = 8\), i.e., the average beat-to-beat variation was <1%. Upon closer examination, inactivation of \(I_{\text{Ca}}\) was slightly faster during the large-amplitude Ca\(^{2+}\) transient (blue trace in Fig. 1A,c), consistent with a more pronounced Ca\(^{2+}\)-dependent inactivation of the current. Furthermore, the tail current observed after repolarization was larger after the large-amplitude alternans Ca\(^{2+}\) transient, consistent with larger Ca\(^{2+}\) release leading to enhanced Ca\(^{2+}\) removal across the surface membrane via NCX.

We addressed the question of whether SR Ca\(^{2+}\) content alternates during Ca\(^{2+}\) alternans with two separate experimental approaches. With the first assay, we rapidly applied 10 mM caffeine after the large and small Ca\(^{2+}\) transient during alternans while recording cytosolic \([Ca^{2+}]\). This approach allowed SR content to be measured with subcellular (subsarcolemmal j-SR vs. central nj-SR) resolution using laser scanning confocal microscopy (Fig. 1B,a). Figure 1B,b shows the average amplitudes of electrically evoked (field stimulation) Ca\(^{2+}\) transients for the j-SR and nj-SR. Average AR was 0.54 ± 0.03 for the jSR and 0.61 ± 0.04 for the nj-SR (\(n = 10\)). As shown in Fig. 1B,c, there were no differences in amplitude of the caffeine-induced Ca\(^{2+}\) transient (i.e., SR Ca\(^{2+}\) load) after the large and small transient, nor were there any differences between the j-SR and nj-SR. These results confirmed earlier measurements in atrial tissue (28).

With the second approach, we measured \([Ca^{2+}]_{\text{SR}}\) levels directly using the low-affinity indicator fluo-5N entrapped in the SR. As shown in Fig. 1B,d (left), the end-diastolic \([Ca^{2+}]_{\text{SR}}\) levels preceding a large SR depletion were identical to the \([Ca^{2+}]_{\text{SR}}\) levels immediately before a small transient (\(n = 9\)). Furthermore, the end-diastolic AR, expressed as the ratio of end-diastolic \([Ca^{2+}]_{\text{SR}}\) before a large and small SR Ca\(^{2+}\) depletion transient, was 1.003 ± 0.002 (Fig. 1B,d (right)), indicating that Ca\(^{2+}\) alternans could occur at constant end-diastolic \([Ca^{2+}]_{\text{SR}}\).

In conclusion, the data shown in Fig. 1 indicate that alternans of cytosolic Ca\(^{2+}\) transients occur in the absence of beat-to-beat alternations of the physiological trigger (\(I_{\text{Ca}}\)) for CICR during excitation-contraction coupling. Furthermore, alternans in end-diastolic \([Ca^{2+}]_{\text{SR}}\) are not required for Ca\(^{2+}\) alternans. Therefore, alternations in \(I_{\text{Ca}}\) and SR Ca\(^{2+}\) content can be excluded as the primary or sole determinants of cytosolic Ca\(^{2+}\) alternans under our experimental conditions. This is consistent with our previous findings in atrial and ventricular myocytes (28, 44). Therefore, in the following experiments, we focused on refractory kinetics and restitution of SR Ca\(^{2+}\) release and tested the hypothesis that alternans of refractoriness of RyR-dependent Ca\(^{2+}\) release is causally linked to cardiac alternans.

Prociposity of spontaneous Ca\(^{2+}\) release during rest after alternans. To evaluate the availability or restitution of the Ca\(^{2+}\)-release mechanism after a Ca\(^{2+}\) transient during alternans, we induced Ca\(^{2+}\) alternans by pacing and measured the time to the occurrence (latency) of spontaneous global Ca\(^{2+}\) release (e.g., in the form of a Ca\(^{2+}\) wave or a spontaneous AP) during a period of rest after pacing. As shown in Fig. 2A,a, the occurrence of spontaneous global Ca\(^{2+}\) release was compared during rest periods after a small Ca\(^{2+}\) transient (left) and a large Ca\(^{2+}\) transient (right). The average latency of spontaneous global Ca\(^{2+}\) release (Fig. 2A,b) was 1.41 ± 0.24 s after the small transient and 2.12 ± 0.42 s after the large transient (\(n = 6, P < 0.05\)). The significantly shorter latency observed after the small Ca\(^{2+}\) transient during alternans suggests that refractoriness of the SR Ca\(^{2+}\)-release mechanism alternates and is
significantly reduced after a small transient (or prolonged after a large transient).

We investigated this hypothesis further by evaluating the frequency of spontaneous elementary SR Ca$^{2+}$-release events [Ca$^{2+}$ sparks (9)] during rest after pacing-induced alternans. Figure 2B,a shows spark recordings after a small transient (left) and after a large transient (right) from the same cell. Spark frequency was twofold higher after the small SR Ca$^{2+}$ transient than after the large SR Ca$^{2+}$ transient (after the small transient: 3.18 ± 0.86 sparks·s$^{-1}$·(100 μm)$^{-1}$ and after the large transient: 1.62 ± 0.75 sparks·s$^{-1}$·(100 μm)$^{-1}$, n = 5, P < 0.002; paired observation). Notably, in the example shown, early after the cessation of pacing a burst of Ca$^{2+}$ sparks was observed after the small transient that was absent after the large transient.

In conclusion, the observation that the latency of spontaneous global Ca$^{2+}$ release and abundance of spontaneous Ca$^{2+}$ sparks during rest after alternans is critically dependent on the time interval between spontaneous Ca$^{2+}$ sparks during rest was preceded by a final small or large-amplitude transient suggests that refractoriness of SR Ca$^{2+}$ release plays a critical role in the Ca$^{2+}$ alternans mechanism. The experiments shown in Fig. 2, however, only indirectly addressed the role of refractoriness for alternans because spontaneous Ca$^{2+}$-release events were evaluated after the cessation of electrical pacing. The following experiments were designed to address this question directly during alternans.

Refractoriness of Ca$^{2+}$ release during alternans. Refractoriness of Ca$^{2+}$ release during alternans was tested by applying premature electrical pulses at exactly defined time intervals during the Ca$^{2+}$ transient induced by a regular pacing protocol. Premature stimuli were applied during large and small Ca$^{2+}$ transients between 100 and 1,000 ms after the regular pulse. The magnitude of Ca$^{2+}$ release induced by the premature stimulus was quantified as fractional release, i.e., the amplitude was normalized to SR Ca$^{2+}$ load in each individual cell to account for cell-to-cell variability of SR Ca$^{2+}$ content. SR load was measured as the amplitude of the Ca$^{2+}$ signal elicited with the rapid application of 10 mM caffeine (the general protocol is shown in Fig. 3A). Figure 3B shows an example where the extra stimulus (black arrowhead) was applied 250 ms after the regular depolarization (filled circles) during alternans, after a small-amplitude Ca$^{2+}$ transient (a), and after a large-amplitude Ca$^{2+}$ transient (b). Release had partially recovered 250 ms after a small transient, whereas after the large transient additional Ca$^{2+}$ release was barely detectable. As shown in Fig.
3C, in response to a stimulus applied after a 500-ms interval, Ca\textsuperscript{2+} release had fully recovered after the small- and almost completely after the large-amplitude alternans Ca\textsuperscript{2+} transients. The results are shown in Fig. 3D. During the small Ca\textsuperscript{2+} transient (dashed lines), SR Ca\textsuperscript{2+} release became partially available in <250 ms, whereas SR Ca\textsuperscript{2+} release during a large transient remained unavailable for >250 ms. The 50% recovery time (taken from the fit to the average data) during the small transient (dashed lines) was 230 ms for the j-SR and 260 ms for the nj-SR. In contrast, the 50% recovery time during the large transient (solid lines) was 350 ms for the j-SR and 450 ms for the nj-SR, i.e., restitution of Ca\textsuperscript{2+} release during the large Ca\textsuperscript{2+} transient was prolonged by nearly 65% and the recovery of release from the j-SR preceded the recovery of nj-SR release by 30–100 ms. The latter is largely explained by the fact that the activation of release in the cell center lagged behind activation in the cell periphery due to the lack of t-tubules in atrial cells and the time required for the propagation of CICR from the cell periphery to the cell center (33, 39, 40, 66). As shown previously, this delay amounts to 45–80 ms (27, 53).

Recovery of SR Ca\textsuperscript{2+} release from inactivation during alternans was further investigated with an experimental approach where step-like increases of [Ca\textsuperscript{2+}], were imposed during alternans using a caged Ca\textsuperscript{2+} compound [DM-nitrophen (31)] and photolytical release of Ca\textsuperscript{2+}. Ca\textsuperscript{2+} released from the SR by uncaging of DM-nitrophen was termed photolysis-induced Ca\textsuperscript{2+} release (PICR). Atrial myocytes were voltage clamped and stimulated with 100-ms depolarization pulses from a holding potential of −40 to +20 mV at frequencies that elicited stable Ca\textsuperscript{2+} alternans. UV (355 nm) laser flashes (lightning symbol) were applied at the beginning of the depolarization pulse (time 0 in Fig. 4A) or at defined intervals ranging from 50 to 400 ms after the beginning of the depolarization pulse (Fig. 4, B and C). This protocol was applied twice to deliver a UV flash of identical delay during a small Ca\textsuperscript{2+} transient (first series, red trace) and a large Ca\textsuperscript{2+} transient (second series, blue trace). The UV laser pulse was preceded by a minimum of 10 depolarization pulses to achieve reproducible Ca\textsuperscript{2+} loading of the SR. In the example shown in Fig. 4A, the UV flash was applied simultaneously with the voltage-clamp pulse. In both series, the amplitude of the transient elicited by the combined application of a depolarization step and UV flash was larger than elicited by membrane depolarization alone (note that depolarization to +20 mV elicited a maximal Ca\textsuperscript{2+} response under identical experimental conditions; data not shown). This demonstrates directly that the physiological trigger of CICR, I\textsubscript{Ca}, does not release all available Ca\textsuperscript{2+} in the SR and that under alternans conditions during the small transient sufficient SR Ca\textsuperscript{2+} would be present, provided there is a sufficiently strong trigger that could overcome the refractoriness of the release mechanism. As shown in Fig. 4A, the amplitude of the Ca\textsuperscript{2+} transient elicited by the combination of membrane depolarization and UV flash was larger after a small transient (i.e., when a large transient is expected, blue trace) than after a large transient (red trace). As shown in Fig. 4A,b, this difference was graded with the AR. Cells with the largest AR of Ca\textsuperscript{2+} transients elicited by membrane depolarization also revealed the largest difference between the Ca\textsuperscript{2+} transient amplitudes elicited by the combined application of depolarization (CICR) and UV flash (PICR). Since end-diastolic [Ca\textsuperscript{2+}], was identical before application of the pulse (red vs. blue trace) and based on our observation that at [Ca\textsuperscript{2+}] \textgreater 300 nM the uncaging Ca\textsuperscript{2+} signal was constant (see METHODS), we could assume that the Ca\textsuperscript{2+} loading of the caged compound was identical and therefore identical amounts of Ca\textsuperscript{2+} were released from the cage despite the fact that [Ca\textsuperscript{2+}], changed during a cytosolic Ca\textsuperscript{2+} transient. Consequently, the difference in [Ca\textsuperscript{2+}], amplitude was the result of differences in PICR.

Figure 4B shows an example where a UV flash was applied 150 ms after the beginning of the depolarization step during a small Ca\textsuperscript{2+} transient (red trace) and a large Ca\textsuperscript{2+} transient (blue trace). The extra amount of Ca\textsuperscript{2+} release by uncaging of Ca\textsuperscript{2+} from DM-nitrophen (PICR) was larger during the small Ca\textsuperscript{2+} transient (ΔF/Fo of PICR was 0.25 for the small Ca\textsuperscript{2+} transient and 0.17 for the large Ca\textsuperscript{2+} transient). Figure 4C shows the effect of the delay of UV flash application on the amplitude of PICR. UV flashes applied during identical Ca\textsuperscript{2+} transients but with increasing delays from the beginning of the transient revealed PICR of increasing magnitudes. This obser-
SR Ca$^{2+}$ RELEASE RESTITUTION DEFINES Ca$^{2+}$ ALTERNANS

Fig. 3. Refractoriness of SR Ca$^{2+}$ release during Ca$^{2+}$ alternans probed by the application of extra electrical stimuli. A: experimental protocol. Extra stimuli were applied at defined intervals (100–1,000 ms) during pacing-induced (2.5 Hz) Ca$^{2+}$ alternans during/after a small (shown) and large alternans Ca$^{2+}$ transient. Filled circles indicate regular field stimulation pulses; extra stimuli are indicated by black arrowheads. In each experiment, SR Ca$^{2+}$ load was subsequently measured by the application of caffeine (10 mM). B and C: subcellular (j-SR, black traces; nj-SR, red traces) Ca$^{2+}$ transients and Ca$^{2+}$ release induced by extra stimuli applied at 250 ms (B) and 500 ms (C) during a small (a) and large (b) alternans Ca$^{2+}$ transient. D: Ca$^{2+}$ release as a function of time interval between a regular stimulus and an extra stimulus, for small (open symbols) and large (filled symbols) transients, and for the j-SR (black) and nj-SR (red). Ca$^{2+}$ release by extra stimuli is expressed as the percentage of fractional release (amplitude of the extra Ca$^{2+}$-release transient divided by the amplitude of the caffeine-induced Ca$^{2+}$ signal). The data shown in D were obtained from a total of 80 myocytes. Δt, time interval between regular electrical stimulus and extra stimulus.
sistently caused stable Ca\(^{2+}\) alternans (Fig. 5A). After stable
alternans had been established, cells were exposed to a low
dose (0.1 mM) of caffeine, which is well established to in-
crease the Ca\(^{2+}\) sensitivity of the RyR (16, 59). As shown in
Fig. 5A, caffeine normalized pacing-induced Ca\(^{2+}\) alternans
within <10 beats. Upon closer inspection, the predominant
effect of caffeine was on the small-amplitude Ca\(^{2+}\) tran-
sient (blue; second series) and a large-amplitude Ca\(^{2+}\) transient (red; first series) and a large-amplitude Ca\(^{2+}\) transient (blue; second series). b, Cor-
relation between AR induced by pacing and
[Ca\(^{2+}\)], signal induced by the combination of
depolarization [Ca\(^{2+}\)]-induced Ca\(^{2+}\) release
(CICR) and UV flash (PICR). B: photore-
lease of caged Ca\(^{2+}\) during small (a) and
large (b) alternans Ca\(^{2+}\) transients (delay:
100 ms) and determination of PICR ampli-
tude. C, three examples of PICR applied with
delays of 100, 160, and 220 ms during depo-
larization-induced Ca\(^{2+}\) transients of identi-
cal amplitude. D: average PICR ratios during
large and small Ca\(^{2+}\) transients (PICR/L/
PICR\(_{a}\)) as a function of the delay between the
depolarization pulse and UV flash. Numbers
in parentheses indicate numbers of cells
tested.

Fig. 5. Effect of ryanodine receptor sensitization on
Ca\(^{2+}\) alternans and refractoriness of Ca\(^{2+}\) release.
A: application of low-dose (0.1 mM) caffeine
normalized pacing-induced Ca\(^{2+}\) alternans. B: sponta-
neous Ca\(^{2+}\) release (solid diamond) during rest in the
presence of caffeine. Shown are subcellular Ca\(^{2+}\) signals from the j-SR (black) and nj-SR (red) (top)
and line-scan images (bottom). Filled circles indicate
electrical field stimulation pulses.
alternans at a pacing frequency of 2.5 Hz and reduced latency on average to 0.71 s (n = 6; data not shown).

Modulation of Ca\(^{2+}\) alternans by step-like increases of \([\text{Ca}^{2+}]_i\). Using photolysis of caged Ca\(^{2+}\) in voltage-clamped myocytes, we tested the effect of rapid step-like elevations of \([\text{Ca}^{2+}]_i\) on alternans threshold pacing frequency and the phase of alternans. UV flashes used to photolysed DM-nitrophen (lightning symbol in Fig. 6) were synchronized with the onset of the depolarization step (filled circles). When UV flashes were applied to cells with stable alternans at the time of an expected small-amplitude transient, two types of responses were observed. In one case, the large Ca\(^{2+}\) transient induced by PICR was followed by a normalization of the Ca\(^{2+}\) transients and the disappearance of alternans while pacing frequency remained unchanged (n = 4; Fig. 6A). In the other case, alternans continued after the PICR-induced Ca\(^{2+}\) transient, but the PICR-induced Ca\(^{2+}\) transient was followed by a small-amplitude transient, indicating a phase shift in alternans (n = 11; Fig. 6B). In contrast, when PICR coincided with a large-amplitude transient during stable alternans, alternans continued without a change in phase (n = 11; Fig. 6C). Finally, in nonalternating myocytes, PICR timed to coincided with a voltage-clamp pulse could initiate alternans at unchanged stimulation frequency (n = 6; Fig. 6D). In summary, these experiments show that interference with Ca\(^{2+}\) cycling during regular pacing by imposing step-like increases in \([\text{Ca}^{2+}]_i\) has profound effects on AR and the alternans phase that will be further discussed below.

**DISCUSSION**

In this investigation, we established direct evidence that beat-to-beat alternans of time-dependent restitution of the SR Ca\(^{2+}\)-release mechanism constitutes a key defining factor of cardiac alternans.

A key element toward a comprehensive understanding of cardiac alternans is the concept of bidirectional coupling of electrical and Ca\(^{2+}\) signaling, leading to the paradigm that alternans are either membrane voltage or [Ca\(^{2+}\)]\(_i\) driven, and alternans has been linked to disturbances in both electrical and Ca\(^{2+}\) signaling (reviewed and discussed in Refs. 29, 51, 63, and 64). Led by the observation, however, that mechanical and Ca\(^{2+}\) alternans can occur in the absence of APD alternans (experimentally confirmed in voltage-clamp experiments) and with constant \(I_{\text{Ca}}\) (10, 14, 28, 62) (see also Fig. 1A in the present study), the focus has shifted (46) toward disturbances in Ca\(^{2+}\) signaling that might underlie alternans and APD alternans may be merely a consequence of Ca\(^{2+}\) alternans. As outlined in the Introduction, here the relationship between fractional SR Ca\(^{2+}\) release and cytosolic Ca\(^{2+}\) sequestration determines whether Ca\(^{2+}\) cycling is stable or undergoes alternans. The key question in this context is how does Ca\(^{2+}\) release during any given heart beat determine Ca\(^{2+}\) release and Ca\(^{2+}\) transient magnitude of the subsequent beat. The recovery of the Ca\(^{2+}\) transient (generally referred to as Ca\(^{2+}\) transient restitution

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Fig. 6. Phase modulation of Ca\(^{2+}\) alternans by PICR. UV flashes (lightning bolts) were applied coincidently with a depolarization pulse in voltage-clamped myocytes during pacing induced Ca\(^{2+}\) alternans (A–C) and during regular Ca\(^{2+}\) transients (D). Filled circles indicate the electrical onset of depolarization pulses (100 ms, to +20 mV from a holding potential of −40 mV). Letters in parentheses indicate the anticipated Ca\(^{2+}\) transient amplitude.
tion) depends on the recovery of the trigger of CICR (\(I_{ca}\)). SR Ca\(^{2+}\) load, and the release mechanism itself (RyRs). These three factors have been addressed directly in the present study.

As shown in Fig. 1A, when \(I_{ca}\) was recorded from voltage-clamped atrial myocytes at stimulation frequencies that induced Ca\(^{2+}\) alternans, no beat-to-beat differences in peak current could be observed. \(I_{ca}\) showed accelerated inactivation kinetics and enhanced tail currents during the large-amplitude Ca\(^{2+}\) transient, consistent with stronger Ca\(^{2+}\)-dependent inactivation and enhanced Ca\(^{2+}\) extrusion via NCX. The lack of alternans of peak \(I_{ca}\) is consistent with our earlier observation (28) and is at variance with observations of alternating L-type \(I_{ca}\) that triggers CICR and Ca\(^{2+}\) alternans (23, 38). Nonetheless, our observations exclude \(I_{ca}\) alternans as the sole cause of cardiac alternans.

Clearly more controversial is the issue whether cardiac alternans require beat-to-beat alternations in SR Ca\(^{2+}\) content as a causative factor (15), and it has been suggested that instability in the beat-to-beat feedback control of SR content leads to Ca\(^{2+}\) alternans (18). We addressed this question experimentally with two different approaches. The first approach relied on measurements of SR Ca\(^{2+}\) load by quantifying the cytosolic Ca\(^{2+}\) signal in response to rapid SR depletion with caffeine. As shown in Fig. 1B, the caffeine-induced Ca\(^{2+}\) transients were identical after the large and small alternans Ca\(^{2+}\) transient, excluding alternating differences in diastolic SR Ca\(^{2+}\) load and confirming earlier findings made in atrial tissue (28). The second approach used a refined method of direct dynamic measurements of [Ca\(^{2+}\)]\(_{SR}\) using a low-affinity Ca\(^{2+}\) indicator (fluo-5N) entrapped in the SR lumen. With this method, we demonstrated reliably that alternating end-diastolic SR Ca\(^{2+}\) content is not required for alternans to occur. These results confirmed our earlier observations in ventricular myocytes, where we showed that while diastolic [Ca\(^{2+}\)]\(_{SR}\) fluctuations can occur during alternans, Ca\(^{2+}\) alternans can also readily occur without significant diastolic [Ca\(^{2+}\)]\(_{SR}\) fluctuations (44), thus also eliminating SR Ca\(^{2+}\) load alternans as a mandatory prerequisite for cardiac alternans.

While APD restitution (including the recovery of L-type Ca\(^{2+}\) channels from voltage- and Ca\(^{2+}\)-dependent inactivation) and sequestering of Ca\(^{2+}\) [specifically reuptake of Ca\(^{2+}\)] into the SR and reestablishment of Ca\(^{2+}\) load (12, 30, 68) have been recognized as contributing factors to electromechanical and Ca\(^{2+}\) alternans, recent findings have provided evidence that refractoriness of CICR or temporary unavailability of RyRs after a release event may contribute to instabilities of cardiac Ca\(^{2+}\) release and vulnerability to arrhythmias (see the discussion in Ref. 56). Experimental studies on the recovery of whole cell Ca\(^{2+}\) transients as well as Ca\(^{2+}\) release at the level of individual Ca\(^{2+}\)-release units (Ca\(^{2+}\) sparks) have preceeding release event (7, 8, 47, 52, 55, 57) have shown that Ca\(^{2+}\) release is unavailable immediately after release due to RyR inactivation and requires several hundred milliseconds for full recovery. These restitution kinetics have raised the possibility that not all of the SR Ca\(^{2+}\) release occurs and that the time-dependent recovery of SR Ca\(^{2+}\) release may be the critical factor for the occurrence of Ca\(^{2+}\) alternans. In the present study, we addressed this question using multiple experimental approaches. We tested four different parameters that are directly or indirectly related to SR Ca\(^{2+}\)-release restitution: the occurrence of Ca\(^{2+}\) sparks and spontaneous global Ca\(^{2+}\)-release events during rest after alternans (indirect parameters) and the availability of SR Ca\(^{2+}\) release elicited at defined intervals during and after a Ca\(^{2+}\) transient in response to a premature electrical stimulus or a step-like increase of [Ca\(^{2+}\)]\(_i\) elicited by photorelease of caged Ca\(^{2+}\). In all these experiments, these parameters were compared between large and small alternans Ca\(^{2+}\) transients. In summary, these experiments provided consistent results showing that during alternans, the restitution kinetics of SR Ca\(^{2+}\) release were prolonged after the large-amplitude transient. Thus, we conclude that time-dependent recovery from refractoriness and the restitution of SR Ca\(^{2+}\) release is a key factor for the generation of Ca\(^{2+}\) alternans. Similar conclusions on the importance of refractoriness of SR Ca\(^{2+}\) release for the occurrence of alternans were drawn from studies on the role of luminal Ca\(^{2+}\) for release in intact mouse hearts (36) and the collective behavior of Ca\(^{2+}\) sparks, where refractoriness of a Ca\(^{2+}\)-release unit after a Ca\(^{2+}\) spark was identified as one of the key determinant factors of cardiac alternans (49).

The nonlinear relationship described earlier between Ca\(^{2+}\) sequestration and fractional SR Ca\(^{2+}\) release that determines the vulnerability to alternans allows the prediction of how endogenous or exogenous perturbances of beat-to-beat Ca\(^{2+}\) cycling affect alternans. As shown in Fig. 5, sensitization of the RyR with low-dose caffeine (0.1 mM) rescued pacing-induced Ca\(^{2+}\) alternans and normalized Ca\(^{2+}\) transient amplitude, an observation also made in ventricular myocytes when alternans occurred as a consequence of spatially fragmented Ca\(^{2+}\) release (38). RyR sensitization to CICR is expected to have complex effects on the propensity for Ca\(^{2+}\) alternans. While low-dose caffeine will initially enhance fractional release, it will also enhance SR Ca\(^{2+}\) leak (4, 16) and thus diminishes Ca\(^{2+}\) sequestration, which favors alternans. The increased SR Ca\(^{2+}\) leak, however, also reduces Ca\(^{2+}\) load and, subsequently, fractional release. The latter would predict a decrease in alternans probability. Evidence in support of the sensitization of RyRs leading to alternans was found in a study (5) on postmyocardial infarction myocytes where hyperactivity of RyRs due to redox modification of the release channel was accompanied by an increased propensity to alternans. In the same study, however, an increase in fractional SR Ca\(^{2+}\) release, a condition favoring alternans, was also found, which potentially could outweigh the effect on increased sensitivity of the release channel. Furthermore, we propose an additional mechanism through which low-dose caffeine normalizes alternans. Sensitization of the RyR accelerates the prolonged restitution kinetics observed after a large-amplitude Ca\(^{2+}\) release during alternans and, therefore, more release channels are available for the next beat and, consequently, the Ca\(^{2+}\) transient amplitude increases. Consistent with our observation, low-dose caffeine has been found to accelerate the time-dependent refractoriness of Ca\(^{2+}\) sparks (47), and such a mechanism is further supported by our observation that low-dose caffeine primarily enhanced the amplitude of the small-amplitude transient and by this mechanism contributed to the normalization of Ca\(^{2+}\) alternans.

Similarly to low-dose caffeine treatment, we demonstrated that in atrial myocytes, \(\beta\)-adrenergic stimulation stabilized pacing-induced Ca\(^{2+}\) alternans. During \(\beta\)-adrenergic stimulation, SERCA activity and, consequently, SR Ca\(^{2+}\) uptake and
load are increased, leading to enhanced fractional release, which tends to promote alternans. Increased SERCA activity, however, also increases the efficiency of Ca\(^{2+}\) sequestration, resulting in protection against alternans. Whether β-adrenergic stimulation favors (13) or protects against alternans and alternans-related arrhythmias, as observed here (see also Ref. 28), depends on which of the β-adrenergic effects predominate (sequestration or fractional release). Consistent with our results are the observations that β-adrenergic stimulation accelerated the restitution of Ca\(^{2+}\) sparks (47), and depletion of the intra-SR buffer calsequestrin resulted in an accelerated releasing of SR Ca\(^{2+}\), a reduced refractoriness of Ca\(^{2+}\) release, and a decreased probability to develop Ca\(^{2+}\) alternans (35).

Extra beats and premature stimuli have been shown to be capable to induce and modulate the phase of cardiac alternans (for a discussion, see Refs. 50 and 63). In a series of experiments, we tested the effect of rapid and large increases of [Ca\(^{2+}\)]; that coincided in time with an electrical stimulus during regular pacing. Several interesting observations were made. A step-like elevation of [Ca\(^{2+}\)], imposed by Ca\(^{2+}\) uncaging applied coincidently with a (expected) small-amplitude Ca\(^{2+}\) transient resulted in two different responses. It either eliminated Ca\(^{2+}\) alternans (Fig. 6A) or led to a phase shift where the next electrically induced Ca\(^{2+}\) transient was of small amplitude instead of the expected large amplitude (Fig. 6B). The latter (Fig. 6B) can be explained by the notion that the combination of electrical stimulation and photolysis of caged Ca\(^{2+}\) led to maximal SR Ca\(^{2+}\) release, leading to prolonged refractoriness. The observation that a similar disturbance (Fig. 6B) could eliminate alternans demands an alternative explanation. We hypothesize that in this case, the elevated [Ca\(^{2+}\)], also increased SERCA (and possibly NCX) activity. Both favor the sequestration of cytosolic Ca\(^{2+}\), which reduces the likelihood of alternans. Whether the cell responds in one way or the other critically depends on the intricate balance among [Ca\(^{2+}\)]. SR Ca\(^{2+}\) load, fractional release, and cytosolic Ca\(^{2+}\) sequestration. When photorelease of Ca\(^{2+}\) coincided with a large transient, the phase of Ca\(^{2+}\) alternans remained unchanged (Fig. 6C). This is explained by the fact that after a large transient refractoriness is already prolonged and the subsequent Ca\(^{2+}\) transient is expected to be of small amplitude. A preceding Ca\(^{2+}\) transient of excessively large amplitude (as induced here by combined CICR and PICR) would be expected to be followed by a transient of even smaller amplitude, and this effect was indeed observed experimentally (Fig. 6C). Finally, combined electrical stimulation and a step-like increase of [Ca\(^{2+}\)], was able to induce Ca\(^{2+}\) alternans (Fig. 6D). We postulate that the additional stimulus imposed by Ca\(^{2+}\) uncaging enhanced CICR and increased fractional release. In this case, the effect of enhanced fractional release dominated over a Ca\(^{2+}\)-dependent enhancement of sequestration. The observation that diastolic [Ca\(^{2+}\)], actually increased after Ca\(^{2+}\) uncaging suggests a reduced net clearance of cytosolic Ca\(^{2+}\). According to our model, the combined result of all these effects was the occurrence of alternans.

In summary, we have demonstrated that beat-to-beat alternans in the time-dependent restitution properties and recovery kinetics of the SR Ca\(^{2+}\)-release mechanism represents a key mechanism underlying cardiac alternans.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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