Virtual electrodes and the induction of fibrillation in Langendorff-perfused rabbit ventricles: the role of intracellular calcium

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Virtual electrodes and the induction of fibrillation in Langendorff-perfused rabbit ventricles: the role of intracellular calcium. Am J Physiol Heart Circ Physiol 295: H1422–H1428, 2008. First published August 1, 2008; doi:10.1152/ajpheart.00001.2008.—A strong premature electrical stimulus (S2) induces both virtual anodes and virtual cathodes. The effects of virtual electrodes on intracellular Ca2+ concentration ([Ca2+]i) transients and ventricular fibrillation thresholds (VFTs) are unclear. We studied 16 isolated, Langendorff-perfused rabbit hearts with simultaneous voltage and [Ca2+]i measurements. Relative to the level of [Ca2+]i before S2 was applied to the left ventricular epicardium, virtual cathode sites for biphasic S2 were associated with lower VFT than biphasic and monophasic S2. The VFT was significantly higher at virtual cathode sites during the first phase of the pulse compared with 25 ± 6% at both virtual cathode-anode and anode-cathode sites for biphasic S2. The VFT was significantly higher and the vulnerable window significantly narrower for biphasic S2 than for either anodal or cathodal S2 (n = 7, P < 0.01). Treatment with thapsigargin and ryanodine (n = 6) significantly prolonged the action potential duration compared with control (255 ± 22 vs. 189 ± 6 ms, P < 0.05) and eliminated the difference in VFT between monophasic and biphasic S2, although VFT was lower for both cases. We conclude that virtual anodes cause a greater increase in [Ca2+]i than virtual cathodes. Monophasic S2 is associated with lower VFT than biphasic S2, but this difference was eliminated by the inhibition of the sarcoplasmic reticulum function and the prolongation of the action potential duration. However, the inhibition of the sarcoplasmic reticulum function also reduced VFT, indicating that the [Ca2+]i dynamics modulate, but are not essential, to ventricular vulnerability.

electrical stimulation; mapping

THE DEMONSTRATION OF VIRTUAL electrode formation after a premature stimulus (S2) given through small epicardial electrodes has provided novel insights into the mechanisms of ventricular vulnerability (7, 15). A virtual cathode is a region with a transient hyperpolarization at virtual cathodes and virtual anodes during the S1 beat, the [Ca2+]i release. In contrast, transient depolarization at virtual anodes during the first phase of the pulse became virtual cathodes during the second phase, and vice versa. Thus a biphasic shock would be expected to have more uniform effects on SR [Ca2+]i, release. If [Ca2+]i transient heterogeneity is important in the mechanisms of ventricular vulnerability, then the induction of ventricular fibrillation (VF) should be easier with monophasic than with biphasic S2. We hypothesize that 1) after a strong S2 stimulus, virtual anodes cause a greater increase in [Ca2+]i than virtual cathodes, and 2) the monophasic S2 is associated with lower VF threshold (VFT) than biphasic S2, but this difference is eliminated by the inhibition of SR function. The purpose of the present study was to test these hypotheses by simultaneously mapping Vm and [Ca2+]i transients at virtual cathodes and virtual anodes during monophasic and biphasic epicardial point (near field) stimulation in Langendorff-perfused rabbit ventricles.

METHODS

The research protocol was approved by the Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center and followed the guidelines of American Heart Association.

Tissue preparation. New Zealand White rabbits (n = 18) weighing 3–5 kg were used in this study. The rabbits were anesthetized. The hearts were quickly removed. The ascending aorta was cannulated and defibrillation shocks to rabbit ventricles (2). In addition to the effects on Vm, a strong electrical stimulus can also significantly change the intracellular Ca2+ concentration ([Ca2+]i) (4). The magnitude and the spatial distribution of these changes are in part determined by the timing of the shock (10). Because heterogeneous postshock [Ca2+]i changes are important in the mechanisms of ventricular defibrillation (6), it is important to study the differential effects of virtual anodes and cathodes on [Ca2+]i, release to better understand the mechanisms of reinduction of VF following defibrillation. As illustrated in Fig. 1, transient hyperpolarization at virtual anodes is expected to increase the driving force for Ca2+ entry through L-type Ca2+ channels (Fig. 1A), potentiating sarcoplasmic reticulum (SR) Ca2+ release. In contrast, transient depolarization at virtual cathodes is not expected to exert this effect (Fig. 1B), resulting in lesser SR Ca2+ release. The magnitude of change is increased with the increasing coupling interval (a, b, and c). These opposite effects are expected to increase the heterogeneity of [Ca2+]i distribution. In contrast, after a biphasic S2, the sites serving as virtual anodes during the first phase of the pulse became virtual cathodes during the second phase, and vice versa. Thus a biphasic shock would be expected to have more uniform effects on SR [Ca2+]i, release. If [Ca2+]i transient heterogeneity is important in the mechanisms of ventricular vulnerability, then the induction of ventricular fibrillation (VF) should be easier with monophasic than with biphasic S2. We hypothesize that 1) after a strong S2 stimulus, virtual anodes cause a greater increase in [Ca2+]i than virtual cathodes, and 2) the monophasic S2 is associated with lower VF threshold (VFT) than biphasic S2, but this difference is eliminated by the inhibition of SR function. The purpose of the present study was to test these hypotheses by simultaneously mapping Vm and [Ca2+]i transients at virtual cathodes and virtual anodes during monophasic and biphasic epicardial point (near field) stimulation in Langendorff-perfused rabbit ventricles.

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secured for retrograde perfusion with warm (36.5 ± 0.5°C) and oxygenated Tyrode solution at a rate of 30–40 ml/min. The composition of the Tyrode solution (in mmol/l) at a pH of 7.4 ± 0.05 was 125 NaCl, 4.5 KCl, 1.8 NaH2PO4, 24 NaHCO3, 1.8 CaCl2, 0.5 MgCl2, and 5.5 glucose and 50 mg/l albumin in deionized water. The coronary perfusion pressure was regulated and maintained at 80 cmH2O, and the hearts were exposed to the air.

**Stimulation protocol.** Two stimulating electrodes were placed on the left ventricle for baseline pacing (cathodal S1) at a cycle length of 350 ms and for monophasic or biphasic S2 stimulation 10–12 mm away using a World Precision Instrument (Sarasota, FL) model A385R constant current isolator. The line connecting S1 and S2 sites was parallel to the epicardial fiber orientation. The stimulating electrodes were made of stainless steel wires that were Teflon-coated except at their tips and had a diameter of 0.20 mm. We performed an in vitro testing and confirmed that a maximum of 60 mA of current can be delivered to the rabbit hearts with this stimulation system. After eight S1 stimulations, a S2 was applied with a stimulus strength representing the net change of \([\text{Ca}^{2+}]_i\) transient at virtual anodes but not at virtual cathodes. The potentiation of SR \(\text{Ca}^{2+}\) release at virtual anodes is also greater at longer stimulus (S2) coupling intervals (a, b, and c in A and B) when SR \(\text{Ca}^{2+}\) release channels have had more time to recover from refractoriness. C: methods for measuring \(\Delta[\text{Ca}^{2+}]_i\). The difference of the \([\text{Ca}^{2+}]_i\) amplitude (\(\Delta\)) was measured 40 ms after the S2.

**Data analysis.** We assumed that the S1-induced action potential has a resting \(V_m\) of −75 mV and a maximum phase 0 upstroke of +15 mV. The fluorescent intensity of \([\text{Ca}^{2+}]_i\), transient was normalized from 0 (end diastolic) to 1 (peak systolic). The \(\Delta[\text{Ca}^{2+}]_i\), amplitude, representing the net change of \([\text{Ca}^{2+}]_i\), after S2 stimulus compared with 7th S1-paced beat, was measured 40 ms after S2 (Fig. 1C). We chose to measure 40 ms after S2 because 40 ms was sufficiently long to allow \(\text{Ca}^{2+}\)-induced SR \(\text{Ca}^{2+}\) release to occur, but sufficiently short to prevent measuring the secondary \([\text{Ca}^{2+}]_i\), rise triggered by a propagated action potential in case the S2 captured the ventricle.

**RESULTS**

*Virtual electrodes and \([\text{Ca}^{2+}]_i\), after a monophasic and biphasic S2.* Consistent with previous studies (8, 11, 15), our optical recordings showed coexistence of virtual anode and virtual cathode after monophasic S2 in all hearts studied. Fig. 2A shows virtual electrode formation after a monophasic anodal S2. The \(V_m\) map shows a dog bone-shaped virtual anode perpendicular to the diastole starting with 240-ms S1-S2 coupling interval with 10-ms decrements at a fixed S2 strength. A defibrillation shock was used to terminate VF. The vulnerable window for that S2 strength was defined as the duration between the shortest and the longest coupling interval that induced VF. After the shortest and the longest S1-S2 coupling intervals were determined, we did not apply S2 at the in-between S1-S2 coupling intervals to minimize the fibrillation/defibrillation episodes that might cause tissue damage. We defined the area on the strength-interval curve associated with the induction of VF as the area of vulnerability. In 6 out of 10 hearts, we administered 1 µM ryanodine and 100 nM thapsigargin to inhibit SR \(\text{Ca}^{2+}\) cycling. The VF was determined again by scanning the S1-S2 coupling interval starting from 80 ms with 10-ms increment and starting from 340 ms with 10-ms decrement at a fixed S2 strength for the shortest and the longest coupling interval that induced VF, respectively. The S2 strength varied from 1 to 60 mA.

**Optical mapping.** Eight hearts were labeled for simultaneous dual optical mapping of \(V_m\) and \([\text{Ca}^{2+}]_i\), according to methods described in detail elsewhere (9). A \(\text{Ca}^{2+}\)-sensitive dye (0.5 mg Rhod-2 AM, Molecular Probes) was infused into the heart over a 10-min period. This was followed in 15 min by a direct injection of a voltage-sensitive dye (RH 237, Molecular Probes) into the perfusion system with 10–20 µl of the 1 mg/ml solution dissolved in dimethyl sulfoxide (1). The hearts were illuminated with a solid-state, frequency-doubled laser (Verdi, Coherent). The fluorescence was acquired simultaneously with two charge-coupled device cameras (CA-DI-0128T, Dalsa) at 4 ms/frame. The digital images (128 × 128 pixels) were gathered from the epicardium of the left ventricle (20 × 20 mm² area) at 1,000 frames continuously with a 12-bit resolution. Optical signals were processed with both spatial (3 × 3 pixels) and temporal (3 frames) filtering. We used a grid to calibrate the locations of the field of view of these two cameras. Using this calibration, we can compare the recordings of \(V_m\) and \([\text{Ca}^{2+}]_i\), from the same locations. Cytochalasin D (5 µmol/l, Sigma) was added to the perfusate to inhibit muscle contraction.
fiber orientation and two virtual cathodes located parallel to the fiber orientation. The simultaneous \([\text{Ca}^{2+}]_{i}\) map shows \([\text{Ca}^{2+}]_{i}\) elevation around the S2, corresponding to the dog bone-shaped virtual anode, but no significant \([\text{Ca}^{2+}]_{i}\) elevation at the surrounding virtual cathodes. Figure 2B shows \(V_m\) and \([\text{Ca}^{2+}]_{i}\) tracings from virtual anode and virtual cathode sites, respectively. At 40 ms after the S2 (red arrowheads in the \([\text{Ca}^{2+}]_{i}\) trace), before the secondary \([\text{Ca}^{2+}]_{i}\) transient elicited by propagated action potentials from the S2 (blue arrowheads in the \(V_m\) trace), \([\text{Ca}^{2+}]_{i}\) was elevated at the virtual anode site, but not at the virtual cathode site.

An activation isochronal map of the propagated response induced by S2 is shown on the right.

In comparison, a monophasic cathodal S2 induced a pair of virtual anodes parallel to the fiber orientation and a dog bone-shaped virtual cathode transverse to the fiber orientation (Fig. 2C). At 40 ms after S2, there was larger \([\text{Ca}^{2+}]_{i}\) elevation at virtual anode than at virtual cathode sites. The region of \([\text{Ca}^{2+}]_{i}\) elevation corresponded directly to the shape of the virtual anode region. Figure 2D shows \(V_m\) and \([\text{Ca}^{2+}]_{i}\) tracings from virtual anode and virtual cathode, respectively. There was
[Ca$^{2+}$], elevation 40 ms after S$_2$ (red arrowheads) at the virtual anode site but not at the virtual cathode site. The [Ca$^{2+}$] elevation at virtual anode occurred before the peak of propagated $V_m$ (blue arrowheads). An activation isochronal map of the propagated response induced by S$_2$ is shown on the right.

Figure 3 shows the effects of an anodal-cathodal biphasic S$_2$ (anodal pulse in the first phase and cathodal pulse in the second phase). Figure 3A shows that the virtual electrode polarizations induced by the first phase of S$_2$ reversed polarity immediately following the second phase of S$_2$. The [Ca$^{2+}$] map 40 ms after S$_2$ showed [Ca$^{2+}$], elevation around the site of the S$_2$, but the shape did not resemble either the virtual anode or virtual cathode regions on the $V_m$ map, and the size of [Ca$^{2+}$] elevation by anodal-cathodal S$_2$ was smaller than that by anodal S$_2$ shown in Fig. 2A. Figure 3B shows $V_m$ and [Ca$^{2+}$] tracings from a virtual anode-cathode site and a virtual cathode-anode site, respectively. There was similar [Ca$^{2+}$], elevation 40 ms after S$_2$ (red arrow) at these two sites. The [Ca$^{2+}$], elevation at either site (red arrowheads) occurred before the peak of propagated $V_m$ (blue arrowheads). An activation isochronal map of the propagated response induced by S$_2$ is shown on the right.

Figure 3, C and D, shows the patterns of [Ca$^{2+}$], and $V_m$ when the biphasic waveform of the S$_2$ was switched to the first-phase cathodal and second-phase anodal (cathodal-anodal). An activation isochronal map of the propagated response induced by S$_2$ is shown on the right of Fig. 3D. When compared with the effects of the anodal-cathodal S$_2$ (Fig. 3A), the virtual electrodes reversed their polarity during the two phases (Fig. 3A). Figures 4 and 5 summarize the average changes in the level of [Ca$^{2+}$], for both monophasic and biphasic S$_2$ delivered over a range of S$_1$-S$_2$ coupling intervals. Monophasic cathodal and anodal S$_2$ elevated [Ca$^{2+}$], to a substantially greater extent at virtual anode sites compared with virtual cathode sites (Fig. 4), and the differences were larger with longer S$_1$-S$_2$ coupling intervals. In contrast, these differences were greatly attenuated with anodal-cathodal or cathodal-anodal biphasic S$_2$ shocks (Fig. 5).

**Different areas of vulnerability of monophasic S$_2$ and biphasic S$_2$.** Figure 6 shows the typical areas of vulnerability of monophasic and biphasic S$_2$ from one heart. The black squares show S$_2$ stimulus that induced VF, whereas the white squares show S$_2$ stimulus that failed to induce VF. No S$_2$ was given at the location marked by cross marks. These graphs show that the areas of vulnerability were much larger for monophasic than biphasic S$_2$ in this heart. Both monophasic anodal and cathodal S$_2$ induced VF in all 10 rabbits tested. However, VF was not inducible by either biphasic anodal-cathodal and cathodal-anodal S$_2$ at any S$_2$ strength in 3 of 10 hearts. In the remaining seven hearts, VF was inducible by both monophasic and biphasic S$_2$. The VFTs in these seven rabbits for monophasic S$_2$ were 23 ± 5 (anodal) and 19 ± 7 mA (cathodal) [P = not significant (NS)]. For biphasic S$_2$, the VFTs were 44 ± 10 (anodal-cathodal) and 40 ± 15 mA (cathodal-anodal) (P = NS). However, the differences between monophasic VFTs and biphasic VFTs were statistically significant (P < 0.01) for all comparisons.

**Figure 4.** $\Delta$[Ca$^{2+}$], amplitudes between virtual anodes and virtual cathodes at different S$_1$-S$_2$ coupling intervals. A: $\Delta$[Ca$^{2+}$], amplitude (net increase in [Ca$^{2+}$], by S$_2$ stimulation compared with 7th S$_1$-paced beat) at virtual anode and virtual cathode when S$_2$ was monophasic anodal. The $\Delta$[Ca$^{2+}$], amplitudes progressively and significantly increased with increasing coupling interval. B: $\Delta$[Ca$^{2+}$], amplitude at virtual anode (white bars) and virtual cathode (black bars) induced by monophasic cathodal S$_2$. *P < 0.01 when compared with $\Delta$[Ca$^{2+}$], amplitude at virtual cathode. #P < 0.01 and †P < 0.01 when compared with $\Delta$[Ca$^{2+}$], amplitude of virtual anode and virtual cathode, respectively, at the S$_1$-S$_2$ coupling interval of 150 ms.

**Figure 5.** Comparison of $\Delta$[Ca$^{2+}$], amplitudes between virtual anode-cathode and virtual cathode-anode after biphasic S$_2$ stimulation. A: $\Delta$[Ca$^{2+}$], amplitudes after anodal-cathodal biphasic S$_2$. There was no significant difference of $\Delta$[Ca$^{2+}$], amplitudes between the 2 virtual electrode sites. However, there was progressive increase in $\Delta$[Ca$^{2+}$], amplitude with increasing S$_1$-S$_2$ coupling intervals. B: similar results of $\Delta$[Ca$^{2+}$], amplitude after cathodal-anodal biphasic S$_2$. The $\Delta$[Ca$^{2+}$], amplitude in both A and B progressively increased with increasing S$_1$-S$_2$ coupling intervals but did not show a significant difference between virtual anode-cathode (white bars) and virtual cathode-anode (black bars). #P < 0.01 and †P < 0.01 when compared with $\Delta$[Ca$^{2+}$], amplitude of virtual anode-cathode and virtual cathode-anode, respectively, at the S$_1$-S$_2$ coupling interval of 150 ms.
In contrast to VFT, the upper fibrillation threshold (UFT) did not show a significant difference between monophasic and biphasic S2. The UFT is defined by the highest stimulus strength that induced VF during the vulnerable period. VF was induced by 60-mA monophasic anodal or cathodal S2 shocks in all rabbits tested, indicating that UFT was \( \geq 60 \) mA. Similarly, the UFT for anodal-cathodal S2 was \( \geq 60 \) mA in all seven hearts, and the UFT for cathodal-anodal S2 was \( \geq 60 \) mA in all seven hearts except for one of which UFT was 50 mA. Therefore, we were not able to compare the mean UFT between monophasic and biphasic S2.

The shortest coupling interval that induced VF averaged 130 ± 21, 120 ± 24, 151 ± 18, and 149 ± 21 ms for anodal, cathodal, anodal-cathodal, and cathodal-anodal S2 shocks, respectively, and the longest coupling interval that induced VF averaged 177 ± 17, 183 ± 18, 180 ± 8, and 181 ± 22 ms, respectively. In seven hearts, VF was inducible by both monophasic and biphasic S2. In these hearts, the maximum width of the vulnerable window [the difference of the longest and the shortest S1-S2 coupling interval that induced VF was 46 ± 18 for monophasic anodal S2 and 74 ± 25 ms for monophasic cathodal S2 \( (P < 0.01) \)]. The maximum width of the vulnerable window for biphasic S2 were 29 ± 12 (anodal-cathodal) and 29 ± 29 ms (cathodal-anodal, \( P = \text{NS} \)). The differences between vulnerable windows of the monophasic S2 and biphasic S2 were statistically significant \( (P < 0.01) \) for all comparisons. These findings indicate that the area of vulnerability is smaller for biphasic S2 than for monophasic S2.

**Effects of thapsigargin and ryanodine on area of vulnerability.** Pretreatment with thapsigargin and ryanodine largely eliminated the \([Ca^{2+}]_i\) transient, so that \([Ca^{2+}]_i\) mapping was not feasible. Thapsigargin and ryanodine treatment significantly prolonged the action potential duration (APD) compared with control (255 ± 22 vs. 189 ± 6 ms, \( P < 0.05 \)). Thapsigargin and ryanodine eliminated the differences in VFT between monophasic and biphasic S2 shocks. Figure 7 shows area of vulnerability of monophasic and biphasic S2 shocks after thapsigargin and ryanodine from the same tissue shown in Fig. 6. The area of vulnerability widened after the treatment. Of note, there was a significant reduction of VFT \( (P < 0.01) \) after the treatment compared with baseline (monophasic anodal S2, 5.8 ± 2.0; monophasic cathodal S2, 6.7 ± 2.6; biphasic anodal-cathodal S2, 6.7 ± 2.6; and biphasic cathodal-anodal S2, 6.7 ± 2.6 mA), but no significant differences of VFT were detected among these four types of S2. The UFT was \( \geq 60 \) mA for both monophasic and biphasic S2 in all rabbits tested. The shortest coupling interval that induced VF after thapsigargin and ryanodine was similar (127 ± 19, 127 ± 21, 135 ± 26, and 128 ± 18 ms for anodal, cathodal, anodal-cathodal, and cathodal-anodal S2, respectively) to baseline; however, the longest coupling interval increased significantly \( (P < 0.01, \text{respectively}) \) after treatment (232 ± 64, 237 ± 34, 223 ± 36, 226 ± 36 ms).
and 228 ± 42 ms for anodal, cathodal, anodal-cathodal, and cathodal-anodal S2, respectively).

**DISCUSSION**

The primary findings of this study are as follows: 1) a monophasic S2 elevates [Ca2+]i, to a greater extent at virtual anodes than at virtual cathodes; 2) by switching the polarity in the middle of the pulse, a biphasic S2 attenuated the differences of [Ca2+]i, and increased VFT; and 3) the depletion of SR Ca2+ stores with ryanodine and thapsigargin eliminated the VFT differences between monophasic and biphasic S2. However, these drugs also increased APD and decreased VFT for both monophasic and biphasic S2.

**Mechanism of increased S2-induced [Ca2+]i transient heterogeneity by monophasic versus biphasic stimulus.** Figure 1 shows that at virtual anodes, the negative deflection of the membrane potential during the S2 increases the driving force (Ca2+ equilibrium potential −Vm) for Ca2+ entry through L-type Ca2+ channels, thereby potentiating SR Ca2+ release. At virtual cathodes, however, further membrane depolarization toward the Ca2+ equilibrium potential decreases the net driving force, resulting in failure to potentiate SR Ca2+ release to the same degree. For monophasic S2, this leads to heterogeneous [Ca2+]i, elevation between the virtual anode and cathode regions. For biphasic S2, on the other hand, virtual anodes during the first phase become virtual cathodes during the second phase, and vice versa, so that the effect on SR Ca2+ release is more balanced. Another theoretical possibility, especially for a relatively late S2, is that membrane depolarization at the virtual cathode could reactivate recovered L-type Ca2+ channels, potentiating further SR Ca2+ release. However, we did not observe potentiation of the [Ca2+]i transient at virtual cathodes in this study.

**SR Ca2+ cycling and ventricular vulnerability.** Coinciding with the greater [Ca2+]i heterogeneity induced by monophasic versus biphasic S2, we found that the VFT was significantly lower for monophasic S2 than for biphasic S2. Other investigators have demonstrated that enhancing L-type Ca2+ channel activity or α1-stimulation can reduce VFT (12, 16). These findings also suggest that SR function plays an important role in modifying the ventricular vulnerability. By inhibiting SR Ca2+ transient with thapsigargin and ryanodine, the APD increased significantly and the differences in VFT between monophasic and biphasic S2 disappeared. Our findings are consistent with the hypothesis that the [Ca2+]i transient heterogeneity is an important determinant of VFT. The relationship between [Ca2+]i and ventricular vulnerability can be partially explained by the bidirectional coupling between APD and [Ca2+]i, (14). It is possible that the [Ca2+]i heterogeneity modulated the vulnerability to S2 through its influence on APD heterogeneity. A second possible explanation to link between [Ca2+]i, and vulnerability is through afterdepolarization and triggered activity. Our laboratory (5) recently demonstrated the presence of [Ca2+]i prefluoresent at the entrance of the central common pathway of S2-induced figure-eight reentry. These findings suggest early afterdepolarization as a possible mechanism of impulse initiation at that site. Consistent with these hypotheses, the inhibition of the SR function by thapsigargin and ryanodine eliminated the differences between monophasic and biphasic S2 on VFT. However, because SR inhibition did not eliminate ventricular vulnerability, these data also indicate that although the Ca2+2 dynamics modulate vulnerability it is not the sole determinant of vulnerability to VF by a strong S2 stimulus.

**Shock-induced [Ca2+]i changes in cultured neonatal rat myocytes.** Fast et al. (3) reported that in cultured neonatal rat myocytes, short-coupled shocks may transiently reduce [Ca2+]i, at sites of both positive and negative Vm changes. Raman et al. (10) extended the observations by evaluating time-dependent changes of shock on Vm and [Ca2+]i, in cultured neonatal rat cells. These results appear to be opposite to those obtained by us. However, those conclusions were based on the analyses of [Ca2+]i changes during the shock, whereas we analyzed the [Ca2+]i changes 40 ms after S2 stimulation. The example shown in Fig. 2 of the study by Raman et al. (10) in fact showed [Ca2+]i elevation in the postshock period. Furthermore, the postshock [Ca2+]i elevation was larger after an anodal shock than after a cathodal shock, and there was more [Ca2+]i elevation after a longer coupling interval than after a shorter coupling interval. Those postshock changes of [Ca2+]i, transient were consistent with the results of the present study.

**Limitations.** This study was performed using small epicardial wires to give S2. Because the stimulation electrodes are small, the current flow through these electrodes is insufficient in achieving defibrillation. Therefore, the results of these studies might not be applicable to defibrillation shocks. [Ca2+]i, and fluorescence do not have a linear relationship. Therefore, the absolute values shown in Figs. 4 and 5 may not reflect the correct Δ[Ca2+]i levels at all coupling intervals. However, the relative values of [Ca2+]i, induced by anodal and cathodal stimuli and by different S1-S2 coupling intervals remain valid. This limitation therefore does not invalidate the conclusion of the study.

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