Spatiotemporal Relationship Between Intracellular Ca\(^{2+}\) Dynamics and Wave Fragmentation During Ventricular Fibrillation in Isolated Blood-Perfused Pig Hearts

Mark Warren, José F. Huizar, Alexander G. Shvedko, Alexey V. Zaitsev

Abstract—Normal “master–slave” relationship between the action potential (AP) and intracellular Ca\(^{2+}\) transient (CaT) is sometimes altered during ventricular fibrillation (VF). The nature of AP/CaT dissociation during VF and its role in inducing wavebreaks (WBs) remain unclear. We simultaneously mapped AP (RH237) and CaT (Rhod-2) during VF in blood-perfused pig hearts. We computed AP and CaT dominant frequency (DF) and CaT delay in each AP cycle. We identified WBs as singularity points in AP phase movies and sites of conduction block (CB) as sites where an AP wavefront failed to propagate. We analyzed spatiotemporal relationship between abnormal AP/CaT sequences and CB sites. We used a calcium chelator (BAPTA-AM) to abolish CaT and test its involvement in WB formation. During VF, the DF difference between AP and CaT was <10% of the respective values in 95% of pixels, and 80% of all CaT upstrokes occurred during the initial 25% of the excitation cycle. Aberrant sequences of AP and CaT occurred almost exclusively near CB sites but could be traced to normal wavefront sequences away from CB sites. Thus, apparent AP/CaT dissociation was largely attributable to spatial uncertainty of the absolute position of block of each wave. BAPTA-AM reduced CaT amplitude to 30.5±12.9% of control and the DF of AP from 12.2±1.6 to 10.4±1.3 Hz (P<0.01), but did not significantly alter WB incidence (0.76±0.19 versus 0.72±0.19 SP/mm²). These results do not support presence of spontaneous, non–voltage-gated CaTs during VF and suggest that AP/CaT dissociation is a consequence rather than a cause of wave fragmentation. (Circ Res. 2007;101:e90-e101.)

Key Words: ventricular fibrillation ■ pig heart ■ action potential ■ calcium transient ■ wavebreak
Ca\textsuperscript{2+},\textsuperscript{16} the mechanism of abnormal Ca\textsuperscript{T} events as well as their cause-effect relationship with WBs during VF remain unknown. In this study we sought to address the following questions: (1) What is the spatiotemporal organization of abnormal Ca\textsuperscript{T} waves during VF and, in particular, the sites where the AP and Ca\textsuperscript{T} waves diverge or new Ca\textsuperscript{T} waves appear? and (2) how do the dynamics and spatiotemporal organization of AP waves during VF change when Ca\textsuperscript{T} is abolished by chelating Ca?\

**Materials and Methods**

**Isolated Blood-Perfused Heart**

Animals were used according to NIH guidelines. Hearts were obtained from pigs (15 to 25 kg) of either sex premedicated with ketamine 350 mg, azaperone 80 mg, and atropine 0.5 mg (i.m.), and anesthetized with pentobarbital 20 mg/kg (i.v.). After isolation via midline sternotomy, the heart was Langendorff-perfused with buffer as described previously.\textsuperscript{10} All the outflow of perfused blood was collected for recirculation by means of tubes inserted into the left and right ventricles via the atrial appendages.\textsuperscript{10} After sealing the blood circulation system, the heart was placed in a chamber with a heated water-jacketed transparent glass wall and superfused with warm (37±0.5°C) oxygenated Tyrode solution.

**Optical Recordings**

We simultaneously recorded fluorescence from voltage-sensitive dye RH-237 and Ca\textsuperscript{2+} sensitive dye Rhod-2 similar to method described previously.\textsuperscript{17} Briefly, the red (Rhod-2) and infrared (RH-237) fluorescence signals were separated using a dichroic mirror (634DCLP, Omega Optical), and were further filtered with a 585±20-nm band-pass and a 720-nm long-pass filter, respectively. Two synchronized digital CCD cameras (DALSA, 64×64 pixels, 300 frames/s) were aligned using a grid pattern with an accuracy of 1 pixel. Additionally, to exclude the Ca\textsuperscript{2+} buffering effect of Rhod-2 on VF dynamics, in a different group of hearts we recorded fluorescence of voltage-sensitive dye Di-4-ANEPPS as previously described.\textsuperscript{16} All dyes were excited with green light (532 nm) emitted by a 5-watt DPSS laser (Coherent) and delivered to the mapped area via fiber optics light guide (Edmund Optics). The field of view was approximately 35 mm in diameter and covered the epicardial area of the anterior LV with the upper left corner just viewing the left anterior descending coronary artery. To minimize motion artifacts, the heart was gently pressed against the glass chamber wall during acquisition of a 5-second movie.\textsuperscript{10}

**Experimental Procedures and Protocols**

We used a total of 12 hearts. In all experiments, episodes of VF were induced by a short (∼1 sec) application of 9V DC current. Measurements were performed at least 5 minutes after VF induction to allow steady state conditions to be attained.\textsuperscript{10,18} Other experimental procedures and measurements were different among experiments and are summarized in the Table. We recorded dual AP and Ca\textsuperscript{T} movies during baseline VF in 7 hearts. After defibrillation, BAPTA-AM was delivered to 4 of these hearts, and in 2 additional hearts where we imaged Di-4-ANEPPS fluorescence. In these experiments, VF was reinduced 10 to 15 minutes after BAPTA-AM loading, and data obtained during VF before and after BAPTA-AM loading were compared. A sham procedure was performed in 2 hearts, 1 loaded with RH-237 and Rhod-2 and the other 1 was loaded with Di-4-ANEPPS. In the latter heart, the sham procedure was performed before infusion with BAPTA-AM. Contractions were monitored in 3 hearts perfused with BAPTA-AM and in the 2 sham experiments. To determine the Rhod-2 fluorescence changes caused by the dye internalization and leak out of the cells, in 2 experiments we recorded Ca\textsuperscript{T} fluorescence movies during 2 hours of VF after injection of Rhod-2. The time course of the Rhod-2 signal evolution was essentially the same in the 2 experiments. The average of 2 such curves, normalized to the maximal Ca\textsuperscript{T} amplitude, provided a calibration curve that was used to correct the difference in Ca\textsuperscript{T} amplitude observed before and after BAPTA-AM perfusion.

**Dye and Drug Delivery to the Blood-Perfused Heart**

Di-4-ANEPPS, Rhod-2-AM, RH237, and BAPTA-AM were obtained from Molecular Probes. Stock solution of Di-4-ANEPPS (5 mg/mL in DMSO) was diluted in Tyrode solution in a volume ratio 1:1000, and 30 to 60 mL of the final solution was directly infused into the aortic cannula. Direct injection of Rhod-2 into blood failed to produce a detectable time-varying signal, presumably attributable to absorption of Rhod-2 by blood cells. However, previously Qian et al\textsuperscript{16} were able to detect Ca-sensitive Rhod2-AM signal in blood-perfused rabbit hearts when the dye was delivered during perfusion with Tyrode solution before switching to blood perfusion. Therefore, for optimal delivery of the dyes into the cardiac tissue, the following procedure was developed. Aliquots of RH237 (50 to 100 μL of a 5 mg/mL solution in DMSO) and Rhod2-AM (1 mL of 2 mg/mL solution in DMSO) were dissolved in warm oxygenated Tyrode solution (120 mL). Before dye delivery, perfusion was switched to a warm oxygenated Tyrode solution (120 mL) to clear the blood perfusate. Subsequently, we administered 120 mL of the dye cocktail followed by an infusion of additional 120 mL of Tyrode solution, before returning back to blood circulation. A continuous cardiac perfusion was maintained throughout the entire procedure (2 to 4

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**Table. Summary of Experimental Procedures Performed in 12 Pig Hearts**

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minutes) during which the heart outflow was discarded. BAPTA-AM was delivered in a similar manner. Specifically, we dissolved 1600 μL of the stock solution (25 mg BAPTA-AM in 2 mL DMSO) into 1.5 L of Tyrode solution. The entire volume of solution was circulated through the heart (~10 minutes) and was discarded before switching back to blood circulation. In 2 sham experiments, we repeated exactly the same procedure but without adding BAPTA-AM to the Tyrode solution.

Myocardial Contractility
Given the strong Ca²⁺ buffering properties of BAPTA-AM, we confirmed the drug delivery to the myocardium by assessing the LV contractility. In each experiment we had a collector tubing inserted in the LV to collect a small amount of outflow (3 to 4 mL/min) from Thebesian veins. This outflow kept the LV filled with blood at a constant end-diastolic pressure determined by the height of the maximal elevation of the outflow tubing with respect to the mid-LV cavity (~5 cm water). A pressure gauge was inserted in series with the LV outflow tubing. Measurements were done during sinus rhythm before and after dye infusion, and after 10-minute perfusion with BAPTA-AM or Tyrode solution (sham experiments). The outflow tubing was blocked for several seconds, just enough to switching back to blood circulation. In 2 sham experiments, we repeated exactly the same procedure but without adding BAPTA-AM to the Tyrode solution.

Data Analysis
Both RH237 and Rhod-2 fluorescent signals were filtered in an identical manner as follows. Spatial filtering by weighted averaging of neighboring pixels was performed by convolution with pyramid-shaped kernel (cone width, 3 pixels). In addition, a temporal median filter (length, 3 frames) was applied. To remove drift (if present), we first applied a running average filter with a large kernel size (100 frames) to the signal. The output of this filter which represented slow frequency components (< 1Hz) was subsequently subtracted from the original signal.

Analysis of the recorded fluorescent signals in the frequency domain has been previously described in detail. The dominant frequency (DF) was defined as the frequency corresponding to the highest peak in the power spectrum. Phase movies were generated by applying the Hilbert transform to the AP fluorescent signal. Singularity point (SP) was defined as a point where all phases converge. SP trajectories were determined in phase movies using SCROLL software (Sergey Mironov, Institute for Cardiovascular Research, SUNY at Syracuse). A starting point of a SP trajectory was considered as the site of a new WB. The total number of WBs per 1024 frames (3.41 s) was determined for each movie of VF.

Phase Delay Between AP and Ca₉T
Custom software first detected all robust local minima and maxima in each individual pixel recording of both AP and Ca₉T movies. In each pulse, the amplitude was defined as the difference between the respective local maximum and minimum. The pulses with amplitudes less than 10% of the difference between the global maximum and global minimum in the same pixel were discarded from this analysis. We determined the time of the onset of both the AP and the Ca₉T at the 50% of the upstroke of the respective pulses. The time delay between the AP and Ca₉T upstrokes in each excitation cycle (see Figure 2, inset) was then plotted as a time delay distribution histogram. We also calculated the time delay normalized to the time interval between the onset of the preceding AP and the onset of the following AP and expressed as a percentage.

Identification of Abnormal AP/Ca₉T Wavefront Sequences
We generated wavefront movies of AP and Ca₉T signals by initially determining the wavefront of each wave as the data points belonging to the range between 10% and 90% of the ascending phase of the respective pulses (Figure 3F). The pulses with amplitudes less than 10% of the difference between the global maximum and global minimum in the same pixel were discarded. We then superimposed the AP and Ca₉T wavefronts and coded AP wavefronts with green color, Ca₉T wavefronts with blue, and their overlap with white (see Figure 3F). For each dual wavefront movie, we plotted a series of vertical and horizontal time-space plots (TSPs) at 4-pixel intervals. During normal propagation, the AP and Ca₉T wavefronts visit all pixels in each excitation cycle, the Ca₉T wavefronts lagging behind the AP wavefronts. In this case each TSP should show continuous bands of both AP and Ca₉T wavefronts spanning the entire row or column of pixels. Conversely, an AP band which does not span the entire range of pixels indicates conduction block (CB) at the pixel where it stops. (Topological relationship between CB sites, WBs and reentry are explained diagrammatically in supplemental Figure I, available online at http://circres.ahajournals.org). All vertical and horizontal TSPs were inspected to determine the spatiotemporal relationship between the AP and the Ca₉T wavefronts, and the association between the CB sites and abnormal AP/Ca₉T wavefront sequences. Abnormal wavefront sequences at the periphery (within 4 pixels of edge) were discarded from further analysis.

The 10% rejection threshold used for identification of the AP and Ca₉T impulses could potentially affect the results. Supplemental Figures IV and V indicate that the distribution of Ca₉T delays and the relationship between the AP and Ca₉T wavefronts did not change appreciably when the amplitude rejection threshold for the AP and Ca₉T was varied between 5 and 20%.

Statistical Analysis
Data are presented as mean±SD. Differences between parameters measured before and after BAPTA-AM perfusion were assessed with the paired Student t test. A value of P<0.05 was considered statistically significant.

Results
Dominant Frequency of the AP and Ca₉T During VF
DF maps of AP and Ca₉T were very similar (Figure 1A), and the mean DF across all pixels in AP and Ca₉T maps was not
statistically different (12.57±1.66 versus 12.56±1.57, Figure 1B). Pixel-to-pixel comparison showed that the DF difference between AP and Ca\textsubscript{T} was less than 10% (less than 1 Hz) of the respective values in 95% of pixels. However, the average pixel-to-pixel absolute difference of AP and Ca\textsubscript{T} DF values reached statistical significance despite the fact that it was very small (0.12±0.09 Hz). Thus, the DF distribution of the AP and Ca\textsubscript{T} was not fully identical but the discrepancy was at least an order of magnitude smaller than previously reported (5.02±1.22 Hz).\textsuperscript{14} Accordingly, the pixel-to-pixel cross-correlation coefficient between AP and Ca\textsubscript{T} was not fully identical but the discrepancy was at least an order of magnitude smaller than previously reported (5.02±1.22 Hz).\textsuperscript{14} 

The next question we addressed was whether any consistent phase relationship existed between the onset of the AP and Ca\textsubscript{T}.

**Time Delay Between AP and Ca\textsubscript{T}**

Figure 2 shows histograms of time delay between the AP and Ca\textsubscript{T} upstrokes expressed in the number of frames. It is clear that in all experiments (n=7) the majority of Ca\textsubscript{T} were tightly coupled to the upstroke of the preceding AP. The peak of the distribution occurred either at 2 or 3 frames (6.7 to 10 ms), and in 80% of excitation cycles the Ca\textsubscript{T} delay was at most 8 frames (26.7 ms). We also calculated the Ca\textsubscript{T} delay as the percentage of the excitation cycle where it occurred. The normalized Ca\textsubscript{T} delay had similar distribution (not shown). In particular, in 80% of all excitation cycles, Ca\textsubscript{T} upstroke occurred within the initial 25% of the corresponding cycle. However, in each experiment there was also a thin “floor” in the histogram reflecting a small fraction of Ca\textsubscript{T}s which had an apparently random phase in the AP cycle. Thus, we sought to explain those seemingly “non–voltage-gated” Ca\textsubscript{T} events.
Spatiotemporal Organization of Abnormal AP/CaT Wavefront Sequences Near CB Sites

Omichi et al14 used previously mutual information (MI) as the main tool to quantify the degree of temporal correlation between the AP and CaT. We also performed a limited analysis of MI which is presented in supplemental Figure VII. However, MI does not take into account spatial factors. To investigate the relationship between the AP and CaT wavefronts during VF in both space and time, we constructed dual wavefront movies (see Methods and Figure 3F). Figure 3A shows a snapshot of one such movie with three short-living reentrant circuits (a, b, and c) in the field of view. It is seen that CaT followed AP (green-white-blue sequence) along the paths of the reentrant circuits. Figure 3D shows that the AP and CaT recordings taken from pixels distant from the core of the reentrant circuits (pixels 2 and 4) show a consistent phase relationship between the AP and CaT. Specifically, every AP is followed, after a delay, by one and only one CaT, even though the shape of both the AP and CaT impulses varies from cycle to cycle. In contrast, the recordings taken in the vicinity of the centers of reentrant circuits b and c (pixels 1 and 3) show bursts of a highly discordant and apparently random relationship between the AP and CaT.

For a more systematic analysis of the spatiotemporal relationship between the AP and CaT wavefronts, we carefully inspected series of vertical and horizontal TSPs such as those shown in Figure 3B and 3C. We first identified all the sites of CB in the TSPs. Examples of CBs are marked with red arrowheads in Panels E-I, E-II, and E-III of Figure 3, which show expanded portions of the TSPs presented in Figure 3B and 3C. The topological relationship between sites of CB in TSPs and various reentrant and nonreentrant patterns during VF is explained in supplemental Figure I. In particular, the core area of a spiral wave reentry will manifest itself as an instance of CB once for each half of a rotation. For example, the reentrant circuit b indicated in Figure 3A makes approximately two and a half rotations (not shown). Accordingly, the vertical TSP (see Figure 3B and 3E-I) constructed
for a line crossing the reentrant circuit b (vertical dashed line in Figure 3A) reveals 5 instances of CB (see red arrowheads in Figure 3E-I). In some cases, however, CB sites could not be identified with a reentrant circuit, including incomplete reentry and a pattern in which waves coming from 2 or more different directions converged to a CB area, forming a “propagation sinkhole”. For example, the 3 instances of CB marked with red arrowheads in Figure 3C are associated with a “propagation sinkhole” pattern (not shown).

Regardless of whether or not they corresponded to an identifiable reentry, the CB sites were the predominant locations of dissociation between the AP and CaiT. The TSPs shown in Figure 3 clearly demonstrate that the normal AP/CaiT wavefront sequence (CaiT follows AP) is present everywhere with the exception of the vicinity of the CB sites (red arrowheads in Figure 3E). Close examination reveals that in the center of the core/CB areas the dual AP/CaiT wavefronts coming from different directions converge and overlap in an apparently random manner, such that the exact stop positions of the AP and CaiT wavefronts do not coincide in the same wavefront pair and vary between consecutive wavefront pairs. This results in a poor association between the AP and CaiT signals in those pixels which are close (and when they are close) to the CB sites. For example, the transient AP/CaiT dissociation observed in pixel 1 (dashed rectangle “I” in Figure 3D) corresponds to the time interval when this pixel is close to the core/CB area of the transient reentrant circuit b, which is manifested in the vertical TSP by 5 instances of CB (red arrowheads in Figure 3E-I). Similarly, the longer streak of AP/CaiT dissociation observed in pixel 3 (dashed rectangle “II & III” in Figure 3D) is associated with abundant occurrences of CB in the vicinity of this pixel (red arrowheads in Figure 3E-II and 3E-III). Note that a seemingly non-voltage-triggered CaiT wave observed in pixel 3 and in the vertical TSP near pixel 3 (red cross in Figure 3B, 3D, and 3E-II), can be traced to a master AP wave in the horizontal TSP (red cross in Figure 3C and 3E-III). Supplemental Figure III presents detailed pixel-to-pixel analysis of the AP and CaiT waves in the vicinity of pixel 3. It shows that despite the apparently random sequence of impulses in the AP and CaiT recordings from pixel 3, every deflection in these recordings could be traced in space and back in time to a normal AP/CaiT wave. These observations underscore importance of spatial information for understanding the sources of the AP/CaiT dissociation during VF.

Based on the analysis of dual wavefront representation shown in Figure 3, we found that all abnormal AP/CaiT wavefront sequences could be reduced to 4 distinct types: “solitary CaiT”, “solitary AP”, “AP/CaiT crossover”, and “CaiT breakthrough”. Specifically, a “solitary CaiT” is the pattern where a CaiT wave triggered by AP propagates beyond the site where AP wave stops (ie, CB site). “Solitary AP” is the pattern where AP wavefront is not followed by a CaiT. “AP/CaiT crossover” is the pattern where CaiT wavefront initially triggered by an AP eventually breaks ahead of the AP wavefront. “CaiT breakthrough” is the pattern where a CaiT wavefront appears “de novo” and cannot be traced to any preceding AP wave. Obviously, normal wavefront pattern is the one whereby CaiT wavefront follows the AP wavefront along its entire span at all times. We found that in the vicinity of CB sites the abnormal AP/CaiT patterns were either of solitary CaiT, or solitary AP, or AP/CaiT crossover type. The first 2 types were related to an unequal depth of penetration of the respective AP and CaiT wavefronts into a CB area. Examples of a solitary AP and solitary CaiT can be seen in the second and the third lower waves in Figure 3E-I. AP/CaiT crossover occurred when an AP-triggered CaiT wave apparently short-circuited the CB area, while its master AP wave took a longer path around that CB area. As a result, the CaiT wave transiently appeared ahead of the respective AP wave. Two examples of AP/CaiT crossover can be seen in the first and in the last waves shown in Figure 3E-III. Note that in the respective dual complexes recorded from pixel 3 (the first and the last complexes inside the dashed rectangle “II & III” in Figure 3D), the CaiT upstroke appears to be ahead of the respective AP upstroke (phase inversion), which could lead us to an erroneous assumption of a non–voltage-gated CaiT in the absence of spatial information. Association of such phase inversion with the CB sites was confirmed using an alternative definition of the AP-CaiT phase difference based on Hilbert transform (see supplemental Figure VI for details).

Abnormal AP/CaiT Wavefront Sequences Outside the CB Sites

In rare occasions we observed abnormal AP-CaiT wavefront sequences which could not be associated with CB sites. Figure 4A presents an example of “solitary AP” event (ie, CaiT block) not associated with a CB site (arrows). Figure 4B shows examples of an “AP/CaiT crossover” (left oblique arrow) and a “CaiT breakthrough” (right oblique arrow). “AP/CaiT crossover” patterns not associated with CB sites occurred in areas of slow propagation, so that in a few pixels the starting point of the CaiT upstroke “broke ahead” of the starting point of the AP upstroke for 1 or 2 frames. However, the geometry of the dual wavefronts before and after this temporary event maintained the normal master–slave configuration (not shown). We counted total of 6 apparent “CaiT breakthrough” patterns in all 7 experiments. None of those caused a detectable disturbance (eg, conduction delay or block) in the following AP wavefront. The case shown in Figure 4B and 4C is the only one where CaiT breakthrough immediately precedes an AP wave (small deflection in Ca, signal preceding the AP upstroke denoted with right oblique arrow in Figure 4B). Figure 4C shows selected snapshots from the respective AP/CaiT wavefront movie. Initially (0ms), a normal AP/CaiT wave invades the field of view from the upper-right corner. At 10 ms, the AP wave is blocked immediately beyond the site of the pixel recording. The AP wave rotates around the area of block and propagates further out of the field of view. CaiT wave does not invade the area around the pixel of interest (20 ms). After a silent period (43 ms), there is a small CaiT breakthrough (50 ms), which is immediately followed by an AP breakthrough (53ms). An instant later (60 ms), the AP wave is breaking ahead of CaiT wave toward the left side of the field. Finally, a large AP/CaiT wave incoming from the upper right corner invades the region (66 ms) and fuses with the apparently focal AP wave which
was preceded by the Ca,T breakthrough. A possibility that this unique occurrence of focal Ca,T preceding an AP represents a DAD-like ectopic depolarization is analyzed in the Discussion.

Abnormal AP/Ca,T Events Summary
Figure 5A shows the distribution of different types of abnormal AP/Ca,T wavefront sequences near and far from the CB sites. It can be seen that 90.2% of all abnormal events are associated with the CB sites (“solitary AP”, 43.2%; “solitary Ca,T”, 39.5%; AP/Ca,T crossover, 7.5%). In contrast, only 9.8% of the abnormal events could not be associated with CB sites. Among those the “AP/Ca,T crossover” type was the most abundant (6.6%), followed by cases of “solitary AP” (2.8%). Finally, “Ca,T breakthrough” patterns were extremely rare (0.4%) and, as previously mentioned, they did not have any detectable impact on the subsequent AP wavefronts.

Because the abnormal AP/Ca,T events are predominantly associated with the CB sites, an increase in wave fragmentation should lead to an increase in the abnormal AP/Ca,T events. In general, CB lines are associated with WBs which can be identified as SPs in phase movies (this is explained diagrammatically in supplemental Figure I). Thus, it is not unexpected that there was a significant correlation between the total number of abnormal AP/Ca,T events and the total number of SPs identified in each heart ($R^2=0.90$, $P=0.0013$, $P=0.0013$).
Figure 5B). Note however, that this correlation does not indicate whether abnormal AP/CaT wavefront sequences are a cause or a consequence of wave fragmentation during VF.

VF Dynamics in the Presence of BAPTA-AM
To investigate mechanistically a possibility that the dynamics of Ca cycling contributes to wave fragmentation during VF as previously proposed,14 we buffered Ca with BAPTA-AM. The pixel recording in Figure 6A shows that BAPTA-AM markedly reduced the CaT signal amplitude but did not change the amplitude of the AP complexes. In the presence of BAPTA-AM, the residual level of CaT signal in general was approaching noise level and did not form any propagating waves (supplemental Movie I). Despite virtual abolishment of the CaT signal, the 2 snapshots of AP phase movies during VF before an after BAPTA-AM (Figure 6B) show multiple wavelets flanked by SPs under both experimental conditions (supplemental Movies II and III). Identification of SP in all hearts perfused with BAPTA-AM demonstrated that WB formation was not reduced after chelating Ca. Figure 7A shows a total SP number normalized to the total area before and after BAPTA-AM in all experiments (0.76±0.19 versus 0.72±0.19 SP/mm², P>0.05). Interestingly, BAPTA-AM caused a small but significant reduction in the DF of excitation (12.2±1.6 to 10.4±1.3 Hz, P<0.01, Figure 7B). As a result, SP incidence normalized to DF showed a small increase after BAPTA-AM which however did not reach statistical significance (0.018±0.005 SP/(Hz × mm²) versus 0.021±0.007 SP/(Hz × mm²), P>0.05). Figure 7C shows that BAPTA-AM significantly reduced the maximal amplitude of CaT averaged over the mapped area (to 30.5±12.9% of control, P<0.002). We used the developed pressure in the LV as an index of BAPTA-AM effectiveness independent of fluorescence imaging. Figure 7D shows that BAPTA-AM reduced the LV pressure to 12.2% of control (P<0.001) in hearts loaded with RH237 and Rhod-2, whereas the contractility was practically unaltered in the sham experiments.

Discussion
This study sheds light on the nature of dissociation between the AP and CaT during VF14,24 and its possible role in VF maintenance. Specifically, it shows that abnormal AP/CaT wavefront sequences are restricted to CB sites, giving rise to apparently non–voltage-gated CaT events, which can however be traced to a region outside of the CB area where the AP leads CaT. Further, it shows that buffering CaT with BAPTA-AM does not reduce incidence of WBs. Taken together, these findings speak against the presence of DAD-like depolarizations during VF and diminish the possible role of intrinsic Ca dynamics2 in WB formation.

AP/CaT Coupling During VF
Our data clearly show that during VF CaT closely tracks the AP in the vast majority of VF cycles despite the high excitation frequencies (11 to 15 Hz). The predominant time lag between the AP and CaT upstrokes measured during VF in our study is in the range of previously reported values (11 to 26 ms) in isolated porcine myocytes steadily paced at frequencies 0.5 to 1 Hz.25 Values outside this range during VF were most likely attributable to the spatial effects observed at the CB sites (see Figure 3 in the main text and supplemental Figure III). Nevertheless, we cannot exclude that the delay of CaT with respect to the AP upstroke is altered during activation at high frequencies. In any case, the
normal triggering mechanism of CaiT seem to be largely preserved in our model of VF.

Omichi et al. used MI as a measure of coupling between the AP and CaiT. In particular, these authors reported that during VF the average MI measured as the function of time shift between the AP and CaiT signals in selected locations was not different from a random relationship. Although the main conclusions of our study are not based on the MI measurements, we have performed a limited analysis of MI presented in supplemental Figure VII. We measured $M_{\text{max}}$, which represents the best possible match between the shape of the AP and the CaiT, minimizing the influence of the time delay inherently present between the 2 waveforms. Indeed, the time lag at which $M_{\text{max}}$ was achieved agreed well with the CaiT delay determined by the analysis of the AP and CaiT upstrokes (not shown). On the contrary, averaging MI over a range of time lags different from the optimal one enhances the influence of non-periodic modulations of both AP and CaiT signals, which clearly occur during VF. Although in our experiments $M_{\text{max}}$ was heterogeneous in different pixels (supplemental Figure VII), in all AP/CaiT recordings obtained from each single pixel in 7 experiments, $M_{\text{max}}$ values was higher than the respective values after random shuffling of the AP signal in respective pairs (supplemental Figure VII). To our opinion, these data cast serious doubts with regard to the notion of predominantly random relationship between the AP and CaiT during VF.

Omichi et al. also reported large differences in the DF distribution measured in the AP and CaiT movies, whereas in our experiments the respective DF distributions were very similar. The average pixel-to-pixel absolute difference of AP and CaiT DF values in our study was $0.12\pm0.09$ Hz versus $5.02\pm1.22$ Hz reported by Omichi et al. These discrepancies may be explained by differences in the experimental model, although the species is the same (pig). In particular, Omichi et al. used a low $Ca^{2+}$ concentration in the perfusate ($0.54$ mmol/L) to reduce the motion artifact. For that purpose, we used a short-duration mechanical restraint, but we perfused the heart with blood containing normal $Ca^{2+}$ concentration ($1.8$ mmol/L). In addition, we mapped the LV in a whole blood-perfused heart, instead of an isolated RV preparation perfused with crystalline solution. For these or other reasons, it is possible that in the Omichi et al. study the intensity of wave fragmentation (and hence the incidence of WB and CB) was much larger than in our model, so that the irregular AP/CaiT sequences were the prevailing pattern everywhere in the preparation. Lack of WB incidence statistics in the report by Omichi et al. prevents us from testing this assumption. However, the diversity of the dynamics of electrical waves during VF under different experimental conditions is clearly recognized nowadays leading to a possibility that Cai dynamics during VF may also depend on even subtle differences in the experimental conditions. Thus we should emphasize that the results presented here are specific to VF electrically induced in the intact, normoxemic, blood-perfused porcine heart. This pattern may not be universal in different species and under different experimental conditions. In particular, the degree of coupling may be different in the presence of ischemia or cardiac disease, a common context of VF in the clinical setting.

**Dissociation Between the AP and CaiT Wavefronts at CB Sites**

A tight coupling between the AP and CaiT during cell depolarization was markedly reduced in the vicinity of CB sites. Importantly, the spatial extent of the AP/CaiT dissociation was restricted to the immediate vicinity (1 to 3 pixels or 0.5 to 1.5 mm) of the CB sites. In contrast, Omichi et al.
reported examples of complete dissociation between the AP and CaT waves in much larger area (=30×30 mm, see their Figure 6A). As we mentioned above, we cannot exclude that in Omichi et al14 study the incidence of WB and CB was much larger than in our study, so that the disorganized patterns associated with CB/WB sites were prevalent.

Our systematic analysis of the AP/CaiT phase in space and time revealed that AP/CaiT dissociation at the CB sites was attributable to an apparently random penetration of the AP and CaT waves into the blocked area. A very detailed pixel-to-pixel analysis of one example showed that the uncorrelated waves inside the region of CB could be traced back in time to normal sequences of AP/CaiT waves outside of this region (see supplemental Figure III). These observations suggest that, in our model of VF, the AP/CaiT dissociation is a consequence rather than a cause of wave fragmentation.

The relevance of the observed variable penetration of the AP and CaT waves into CB sites (see Figure 3 in the main text and supplemental Figure III) remains uncertain. It is important to emphasize that the difference in penetration of AP and CaT waves into CB sites were never more than 1 to 3 pixels in size (0.5 to 1.5 mm). This may be at the limit of the effective spatial resolution of conventional wide field, epi-fluorescence optical mapping, which is determined by photon diffusion in the depth of the myocardium.28,29 The integration of both RH237 and Rhod-2 fluorescence signals through subepicardial layers could potentially distort our results. Because RH237 emits at a longer wavelength than Rhod-2, the fluorescence representing the AP signal could have been collected from a larger depth than the fluorescence representing CaT. Larger contribution of deeper layers of tissue to the AP signal as compared with the CaT signal could have affected the surface manifestation of AP/CaiT dissociation. This seems unlikely, however, given that we observed a similar prevalence of “solitary AP waves” and “solitary CaiT waves” (see Figure 5A). Nonetheless, we cannot exclude that the presence of low-amplitude CaT waves at the CB sites was underestimated as compared with AP waves. In addition, spatial integration of the optical signals in our system could have lead to “averaging out” abnormal Ca2+ events at the spatial scale less than approximately 0.5 mm. However, if microscopic Ca2+ responses during VF were predominantly abnormal, it would lead to a global macroscopic dissociation between the AP and CaT waves, which was not the case in our study.

At a more conceptual level, we should note that the nature and fine structure of CB sites during VF is not fully understood. It is customary in the field to use interchangeably terms “conduction block”, “wavebreak”, and “the core of a spiral wave”. However, these are not necessarily the same. Whereas some of CB sites could be identified with a reentrant circuit (and therefore formally corresponded to the “spiral wave core”), other CB sites corresponded to incomplete reentry, and still other CB sites corresponded to “propagation sinkholes” where no reentrant circuit could be identified. The nature of the core of a scroll wave is also far from understood. According to one concept, the spiral wave core is a 2-dimensional area which is at rest, ie, it is “not excited but excitable”. However, another computational study demonstrated that the core of a spiral wave can have a circular or linear shape depending on the parameters of the excitable medium. Experimentally, an excitable core was demonstrated only under very specific conditions of atrial tachycardia, with a single fixed reentrant circuit in the mapped region. However, our own experience (eg, supplemental Figure III) as well as data by others do not provide evidence for core areas which are at rest. Rather, a common observation is the presence of an elongated central area of block which is continuously depolarized because of variable penetration of the reentrant wave toward the center of the blocked area. Regardless of the true nature of the spiral wave core, our data indicate that the penetration of Ca waves inside the spiral core region may be different from penetration of the AP waves. Regenerative Ca waves can propagate through multicellular preparations because of Ca2+-induced Ca2+ release and Ca2+ diffusion between cells. Thus, it is possible that during VF Ca2+ waves might propagate beyond the site where the AP wave is blocked thus contributing to the observed AP/CaiT dissociation. Further progress in understanding the nature of the AP/CaiT dissociation during VF will most likely require spatial resolution beyond the limit of the conventional wide-field illumination optical mapping technique, such as the resolution achievable with confocal imaging systems.

Does Spontaneous Ca2+ Release Play a Role in VF Dynamics?

Intracellular calcium overload-induced spontaneous Ca2+ release from the sarcoplasmic reticulum (SR) causes afterpolarizations which can trigger action potentials and ectopic arrhythmias. In ferret hearts perfused with crystalline solution, pacing induced VF lead to a rapid and marked increase in Ca. Others demonstrated that manipulations leading to Ca overload readily induced VF. Those studies, however, did not provide definitive data as to the role of Ca oscillations in the maintenance of VF (this topic is discussed in Ref. 14). A number of computational studies suggested a possibility for a significant contribution of spontaneous SR Ca2+ release secondary to Ca2+ overload into the destabilization of spiral waves either during the initiation or maintenance of VF, depending on certain parameters of Ca formulations used in those studies. On the experimental side, Omichi et al speculated that “the failure of Ca to faithfully track Vm during VF might be explained by spontaneous Ca-induced Ca2+ release becoming dominant over Ca2+ release triggered by the L-type Ca2+ current during the AP”.

Whereas direct quantitative comparison of our data with numerical and experimental results discussed above is hardly possible, we can discuss the extent at which our data are consistent with the proposed role of spontaneous Ca2+ release during VF. We assert that the following experimental observations would support an active role of spontaneous Ca oscillations in the mechanism of VF: (1) identification of non–voltage-gated Ca events during VF; (2) effects of non–voltage-gated Ca events with respect to the following excitation wavefront; (3) a decrease or elimination of wave fragmentation after abolishment of CaT. Our data does not
support any of these notions. We found only 6 “Ca\textsubscript{T} breakthrough” patterns in all 7 experiments (0.4% of all abnormal Ca\textsubscript{T} events). Neither of those had any detectable influence on the propagation of the following AP wave. In only one case we observed a “Ca\textsubscript{T} breakthrough” which preceded an AP wave (Figure 4), which is topologically compatible with a DAD-like ectopic activation. However, in that case the Ca\textsubscript{T} breakthrough appeared in the area not invaded by either the AP or Ca\textsubscript{T} wave in the previous cycle (Figure 4C). This raises a possibility that the apparent Ca\textsubscript{T} breakthrough in that case is associated with a 3-dimensional reentry, which would make it similar to “AP/Ca\textsubscript{T} crossover” events we observed in clear cases of reentry on the surface (see Figure 3 in the main text and supplemental Figure III). Even if the case in discussion is a true DAD-like event, the rarity of this event speaks against its importance in the maintenance of VF in our model. Finally, we found that abolishing Ca\textsubscript{T} with BAPTA-AM did not alter significantly the number of WBs during VF. BAPTA-AM was previously shown to eliminate Ca\textsuperscript{2+} overload-induced DADs in paced ferret papillary muscles,\textsuperscript{31} and completely inhibited the onset of APD alternans in rapidly paced rabbit myocytes.\textsuperscript{11} Thus, failure of BAPTA-AM to suppress wavebreak speaks against the idea that the dynamics of Ca\textsubscript{2+} fluctuations and the related changes in the APD are major factors of VF maintenance.\textsuperscript{2,13,14}

It is important to emphasize that the results obtained with BAPTA-AM allow us to dissect the relative role of specific components of the Ca\textsubscript{2+} handling system in the VF dynamics. Ca\textsubscript{T} can influence the AP during VF mainly through Ca\textsubscript{2+}\textsubscript{,L}–dependent inactivation of I\textsubscript{Ca,L} and activation of the NCX.\textsuperscript{2} Buffering of the global Ca\textsubscript{2+} concentration with BAPTA-AM should effectively eliminate the feedback mechanism involving the NCX. BAPTA may also eliminate a small component of Ca\textsubscript{2+}–dependent inactivation of I\textsubscript{Ca,L} by the bulk cytosolic Ca\textsubscript{2+},\textsuperscript{42} (which might explain a slight decrease in VF excitation frequency we observed in the presence of BAPTA-AM, see Figure 7B). However, buffering of the global Ca\textsubscript{2+} does not affect the main component of Ca\textsubscript{2+}–dependent inactivation of I\textsubscript{Ca,L} caused by Ca\textsubscript{2+}\textsubscript{,L} influx through I\textsubscript{Ca,L} and by Ca\textsubscript{2+} release from the SR.\textsuperscript{43} In contrast to minor effects on VF caused by buffering Ca\textsubscript{T} with BAPTA-AM in our study, blockade of I\textsubscript{Ca,L} in previous studies led to very significant stabilization of rotors during VF, culminating in complete elimination of wave break and conversion of VF into monomorphic VT.\textsuperscript{2,13,14}

This suggests that, among possible feedback mechanisms between Ca\textsubscript{T} and the AP during VF,\textsuperscript{2,27,44,45} the mechanism involving inactivation of I\textsubscript{Ca,L} predominates over the mechanism involving the NCX.

**Limitations**

In addition to limitations mentioned above in relation to specific topics of the discussion, we should mention the following. Our conclusions are based on dual AP and Ca\textsubscript{T} recordings from the subepicardial layers, and thus we cannot exclude the possibility that in the depth of the tissue or on the endocardium, the relationship between AP and Ca\textsubscript{T} is different from that recorded in the present study. We did not specifically address the role of Ca\textsubscript{2+} cycling during VF initiation, because we focused on the established steady-state phase of VF. Although suppressing of Ca\textsubscript{T} by BAPTA-AM did not prevent immediate induction of VF by a brief application of direct current, we cannot exclude that in different modes of VF initiation, intact Ca\textsubscript{2+} dynamics is more critical for the onset of VF. Lastly, our study was performed in normal hearts. Alteration of Ca\textsubscript{2+} dynamics related to ischemia or heart failure could change the relationship between the AP and Ca\textsubscript{T} during VF. Further studies are needed to resolve these issues.

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**Disclosures**

None.

**References**


Spatiotemporal Relationship Between Intracellular Ca\(^{2+}\) Dynamics and Wave Fragmentation During Ventricular Fibrillation in Isolated Blood-Perfused Pig Hearts
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Methods

Topological relationship between the wavebreak (WB) and conduction block (CB).

Figure S-1, A-D illustrates the topological relationship between the WB and the CB. Although these two entities are functionally related, they are topologically distinct. Fundamentally, both WBs and CB are a result of an interaction between the wavefront and the refractory wavetail of a previous impulse (Fig. S-1, A-B). When the wavefront (WF in Fig. S-1) encounters the wavetail (WT in Fig. S-1), two WBs are formed. The segment of the original wavefront between the two WBs fails to propagate any further, which gives rise to an instantaneous line of CB connecting the two newly born WBs (Fig. S-1,B-C). Note that there is no theoretical limit for the length of a CB line. Therefore, within a limited field of view in our study, we can observe two, one or no WBs associated with a particular CB line. Note also that a CB line is by definition an instantaneous phenomenon (it has a zero life span) and it can be identified only by analysis of the consecutive positions of the activation front, for example using time-space plots (TSPs). In a TSP, an intensity profile along a selected pixel line is plotted versus time yielding a pattern of propagation along the selected line (Fig. S-1D; see also Methods in the main text). In contrast, WBs can have any life span and they can be identified in individual frames of a phase-transformed movie as the point where all phases of the cardiac cycle converge.¹

A CB site identified in a TSP constructed for a particular pixel line is a point of intersection between that pixel line and a line of CB (see Fig. S-1D). Any of the newly formed WBs may or may not give rise to a spiral wave reentry. Given at least one complete rotation, a core of a spiral wave can be defined, which will yield a two-sided “CB area” in TSPs created for those pixel lines which cross the core area. In such TSPs, the core area will manifest itself as an instance of CB once for each half of a rotation, forming a “Christmas-tree” pattern. In a classical case, the waves propagate away from the center of the spiral wave making it a propagation...
source (see Fig. S-1E). However, during VF we also observed (although more rarely) an opposite pattern whereby the waves coming from different directions converged into the center of a CB area, forming a “propagation sinkhole” (see Fig. S-1F). In addition, we frequently observed incomplete reentry where a line of CB was formed but the end(s) of broken wave(s) did not make a complete rotation. Thus, during VF, CB is a more general phenomenon than WB or spiral wave reentry. In our analysis of the spatiotemporal relationship between the AP and CaT (see the main text) we did not make distinction between CB sites related to different kinds of reentrant or non-reentrant patterns.

**AP-CaT phase difference using Hilbert transform**

In order to determine the phase difference between AP and CaT fluorescent signals, we first performed phase transformation of each signal as described previously. Briefly, the instantaneous phase of the AP and CaT signals was determined by transforming the original signals such that every spectral element was shifted by its corresponding quarter cycle (Hilbert Transform). Then, the instantaneous phase was obtained from the inverse tangent of the ratio of the transformed signal to the original signal, yielding a phase signal \( \theta \) in which the full AP or CaT cycle corresponded to a monotonic progression of phase from 0 to \( 2\pi \) (Figure S-2A). The phase difference \( \theta_{\text{AP-CaT}} \) was calculated by subtracting the phase of the CaT signal \( \theta_{\text{CaT}} \) from the phase of the AP signal \( \theta_{\text{AP}} \). In normal AP-CaT sequences (CaT upstroke lagging behind the AP upstroke), \( \theta_{\text{AP-CaT}} \) showed a transient negative deflection with the amplitude close to \(-2\pi\) at the beginning of the excitation cycle (see Fig. S-2, B-D). Conversely, if CaT upstroke was ahead of the AP upstroke, \( \theta_{\text{AP-CaT}} \) showed a transient positive deflection with the amplitude close to \(+2\pi\) at the beginning of the excitation cycle (see examples in Fig. S-5, A and C). Movies of \( \theta_{\text{AP-CaT}} \) were created and color-coded in such a way that the range of \( \theta_{\text{AP-CaT}} \) from \(-2\pi\) to \(+2\pi\) corresponded to color wheel changing from purple to red. Thus, red spots in the frames of \( \theta_{\text{AP}} \).
Mutual information

The mutual information (MI) is a nonlinear measure of the statistical dependence between two variables. We calculated the MI between filtered AP and Ca\textsubscript{T} signals similarly to Omichi et al. However, unlike those authors, we computed MI in all pixels of the mapped area and we used all time points contained in the original signals without decimation (i.e., without increasing the time interval between consecutive data points). Given that MI depends on the time lag between AP and Ca\textsubscript{T}, we computed MI for a range of time lags from 0 to 333 ms which reliably exceeds VF cycle length. From this set of MI values, we selected the maximum value ($M_{\text{max}}$) which represented the best match between the shape of the AP and Ca\textsubscript{T} minimizing the influence of the time delay between the two waveforms. In each experiment, $M_{\text{max}}$ computed for AP and Ca\textsubscript{T} signals were compared to the range of $M_{\text{max}}$ obtained in the same experiment after the AP signal in each pixel was shuffled using a perfect shuffle algorithm.

Results

Detailed analysis of the relationship between the AP and Ca\textsubscript{T} near a CB site.

Figure S-3 of the data Supplement presents a more detailed analysis of the AP and Ca\textsubscript{T} in the vicinity of pixel 3 shown in Figure 3 of the main text. This pixel has coordinates (40, 26) in Fig. S-3. The purpose of this analysis is to show that despite the apparent lack of correlation between AP and Ca\textsubscript{T} in pixel (40, 26), every complex in the dual recordings from this pixel can be traced in space and back in time to a normal AP/Ca\textsubscript{T} wave. Figure S-3A shows the dual signal recording from this pixel with a label for every discernible deflection in AP and Ca\textsubscript{T} signals. We analyzed AP and Ca\textsubscript{T} signals along two perpendicular pixel lines centered at pixel (40,26) as shown in Figure S-3B. In the following description of Figure S-3, we will refer to these
lines as simply the horizontal and the vertical line, and we will refer to pixel (40,26) as the central pixel. Figure S-3C and S-3D show the AP tracings respectively recorded from each pixel along the vertical and horizontal directions. Figure S-3E and S-3F show the corresponding Ca\textsubscript{i}T traces recorded from the same pixels. Solid red lines in Panels C-E denote AP and Ca\textsubscript{i}T waves with amplitudes > 10% of maximum in respective recordings. Dashed red lines denote visually detectable AP and Ca\textsubscript{i}T waves with amplitudes below 10%. In each AP wave, the point where the solid and the dashed lines meet correspond to the formally defined point of CB. It is clear that the position of the CB point is uncertain to the extent at which the chosen amplitude criterion can distinguish between active propagation and electrotonus or graded response. In case of Ca\textsubscript{i}T waves, we used the same 10% criterion to formally define “Ca\textsubscript{i}T wave block”, without assigning any particular physiological meaning to it. Finally, in Figure S-3G and S-3H the lines representing the AP (green) and Ca\textsubscript{i}T (blue) waves are superimposed to visualize their spatiotemporal relationship.

Lower case letters in Figure S-3 indicate AP and Ca\textsubscript{i}T impulses belonging to the same AP/Ca\textsubscript{i}T wave pair. Clearly, AP/Ca\textsubscript{i}T wave pair a is normal in all recordings (Figure S-3, C and H). AP/Ca\textsubscript{i}T waves b-e emerge as independent breakthroughs (AP first) and all converge in the vicinity of the central pixel, forming a “propagation sinkhole” pattern. Specifically, AP/Ca\textsubscript{i}T waves b and c enter the area of interest from the top and from the right, respectively, but are blocked before reaching the central pixel. In contrast, Ap/Ca\textsubscript{i}T wave d comes from the lower-left quadrant yielding a normal sequence of AP and Ca\textsubscript{i}T in the central pixel. AP/Ca\textsubscript{i}T wave e comes from the upper-right quadrant and barely reaches the central pixel with the AP wave making a larger impact than the Ca\textsubscript{i}T wave. AP/Ca\textsubscript{i}T wave f is a single-rotation reentrant wave which circles around the central pixel giving rise to “double potential”-like waveform in the AP recording but a single deflection in the Ca\textsubscript{i}T recording. Interestingly, AP wave g emerges as a breakthrough (followed by Ca\textsubscript{i}T wave) and forms a reentrant circuit that makes four incomplete
rotations \(g_1-g_4\) around the central pixel. During the first rotation \(g_1\) wave \(g\) gives rise to a normal sequence of AP and \(\text{Ca}_\text{T}\) in the central pixel. In contrast, during the second rotation \(g_2\) the \(\text{Ca}_\text{T}\) upstroke in the central pixel occurs before the respective AP upstroke reaches the site, giving the appearance of a Ca breakthrough pattern in the vertical line (Figure S-3C, E, G). This results from the \(\text{Ca}_\text{T}\) wave reaching the central pixel during the first half of the rotation, whereas the AP wave reaches that pixel only during the second half of the rotation, as clearly seen in the horizontal line (Figure S-3D, F, H). Effectively, in this case the \(\text{Ca}_\text{T}\) wave is initially slaved to the AP wave, but then it apparently shortcuts the CB area and transiently gets ahead of its master AP wave which takes a longer path around the CB area. Hence, in this case we have an example of the “AP/\(\text{Ca}_\text{T}\) crossover”.

During the third rotation \(g_3\), the central pixel is right at the pivoting point of the reentry and seems to be continuously depolarized by electrotonic influences from various directions resulting in a series of humps in the AP recording. In contrast, during rotation \(g_3\), the \(\text{Ca}_\text{T}\) wave forms one consolidated impulse in the central pixel. During the fourth rotation \(g_4\), whilst the AP wave is represented in the central pixel by a barely discernable hump, the \(\text{Ca}_\text{T}\) wave is represented by an impulse exceeding the 10% threshold for detection. Thus, this \(\text{Ca}_\text{T}\) impulse \(g_4\) in the central pixel may appear as a non-voltage gated Ca release\(^4\) if only the single pixel recording and the vertical line are considered. Importantly, only by having the additional information from the signals in the horizontal line of pixels, we were able enables us to track this \(\text{Ca}_\text{T}\) wave in space and back in time to its voltage trigger (Figure S-3D, F, H).

Upon termination of the described episode of reentry, the AP and \(\text{Ca}_\text{T}\) waves \(i-j\) and \(l\) emerge as independent breakthroughs at different locations around the central pixel and, similar to waves \(b-e\), form a “propagation sinkhole” around the central pixel. Thus, decremental and variable penetration of the AP and \(\text{Ca}_\text{T}\) waves \(i-j\) and \(l\) into the CB area gives rise to apparently
random relationship between the AP and Ca\textsubscript{T} in the central pixel. Subsequently, AP/Ca\textsubscript{T} waves \textit{k} and \textit{m} form a single-rotation reentrant pattern. The case of wave \textit{m} is interesting because it yields a “double potential”-like waveform in the AP recording but a consolidated deflection in the Cai recording, which gives the appearance of a AP/Ca\textsubscript{T} crossover pattern in the central pixel (Figure S-3C, E, G). Finally, AP/Ca\textsubscript{T} wave-pairs \textit{n} and \textit{o} look perfectly normal heralding dissipation of the CB area which persisted around the central pixel for about ten cycles. Expectedly, Ca\textsubscript{T} followed AP everywhere in waves \textit{n} and \textit{o} (Fig. S-3,G-H). Thus, summarizing the unavoidably long analysis above, Figure S-3 demonstrates that the apparently random relationship of AP and Ca\textsubscript{T} impulses at the CB sites is caused by variable penetration of AP and Ca\textsubscript{T} waves into a CB area as they converge from different directions. This results in abnormal wavefront sequences of “solitary Ca\textsubscript{T}”, “solitary AP”, or “AP/Ca\textsubscript{T} crossover” types. Interestingly, the CB area around the central pixel survived several changes in the pattern of wave propagation around it from a “propagation sinkhole” to reentry and back. This may suggest that all waves from \textit{b} to \textit{m} are surface manifestations of the same three-dimensional scroll wave whose topology and orientation with respect to the epicardial surface changes from cycle to cycle.

The effect of the amplitude rejection threshold on the analysis of spatiotemporal phase relationship between the AP and Ca\textsubscript{T}

Figure S-3 above shows that the amplitude of both AP and Ca\textsubscript{T} impulses gradually decreases in the vicinity of CB sites and therefore the choice of the amplitude rejection threshold could potentially affect the main conclusions of this study. In order to exclude this possibility, in one experiment we compared results using 5%, 10% and 20% threshold criteria. Figure S-4 shows that the Ca\textsubscript{T} time delay histogram was essentially independent on the choice of the rejection thresholds. Figure S-5 shows a TSP of a pixel line from a AP-Ca\textsubscript{T} upstroke movie created using 5%, 10% and 20% rejection threshold. Recall that this TSP shows superimposed
positions of the AP and Ca,T wavefronts along the selected pixel line in consecutive frames (see Methods in the main text). We note first that the wavefronts are represented very similarly at different rejection thresholds. Perhaps, at 5% threshold we start picking some noise (see small features in Fig. S-5, A), whereas at 20% threshold we start losing some relatively large features which most likely represent legitimate wavefronts. This suggests that 10% threshold is close to optimal. It is also clear that the AP and Ca,T wavefronts bear a consistent phase relationship most of the time during VF, irrespective of the selected threshold criteria (e.g., see pixel a in Fig. S-5). However, the actual positions of the CB sites (i.e. the points where the wavefronts are interrupted), depend slightly on the amplitude rejection threshold. Essentially, the lower is the rejection threshold, the farther a particular wavefront extends before its amplitude falls below the rejection threshold, which formally defines the CB site. For example, let us consider pixel a in Fig. S-5. During 3 activations marked with asterisks in Fig. S-5, A-C, there is a two-sided CB area in the vicinity of this pixel, such that wavefronts propagate either below or above this area, but not through. One can see that at 5% threshold the ends of the waves extend deep in the CB area so that the waves from above and below the CB area overlap in pixel a (see Fig. 5A). At 10% threshold there is less overlap (Fig. 5B), and at 20% threshold there is no overlap (Fig. 5C). It is also true that the exact pattern of abnormal AP/Ca,T wavefront sequences associated with CB sites depends slightly on the amplitude rejection threshold. In particular, the relationship between the exact stop positions of the respective AP and Ca,T wavefronts may change depending on the rejection threshold. This can be seen in Panels D and E of Fig. S-5 which show the enlarged portions of TSPs shown in Fig. S-5, A (5% threshold) and B (10% threshold), respectively. Let us consider, for example, the third AP/Ca,T wave at the bottom of Panels D and E in Fig. 5 (underneath the third asterisk). In this wave, at 5% threshold the stop positions of the AP and Ca,T wavefronts are exactly the same (Fig. S-5D). However, at 10% threshold the formal stop position of the third Ca,T wavefront extends one pixel beyond the stop position of the third AP wavefront (Fig. S-5E). However, the overall difference is really minor. It is clear that
the exact stop positions of the respective AP and Ca\textsubscript{i}T waves at CB sites are, overall, random at any reasonable amplitude rejection threshold. This corresponds to a quite remarkable mutual randomness of single pixel AP and Ca\textsubscript{i}T recordings taken during time intervals when those pixels are in the vicinity of a CB site (see pixels a and c in Fig. 5F). It would be very tempting to conclude that there is no association between the AP and Ca\textsubscript{i}T during VF if one would consider only this type of single site recordings. However, the analysis of the spatiotemporal organization of the AP and Ca\textsubscript{i}T wavefronts in the large number of pixels clearly indicate that the random relationship between the AP and Ca\textsubscript{i}T is restricted to the CB sites and this observation is robust with respect to the amplitude rejection threshold chosen for detection of individual AP and Ca\textsubscript{i}T impulses.

**Analysis of spatiotemporal phase relationship between the AP and Ca\textsubscript{i}T using \(\theta\text{AP-CaiT}\)**

An alternative approach to study the spatiotemporal phase relationship between the AP and Ca\textsubscript{i}T is to use the phase difference \(\theta\text{AP-CaiT}\) based, for example, on Hilbert Transform as described in the Data Supplement Methods above. (We should note that phase transformation using time-embedding algorithm\textsuperscript{1} yields very similar results). A perceived advantage of \(\theta\text{AP-CaiT}\) analysis is that it relies exclusively on the intrinsic time dependence imbibed in the fluorescent signals and does not involve threshold criteria. Figure S-6 shows the results of \(\theta\text{AP-CaiT}\) analysis applied to the same data as presented in Fig. S-5. Panels A-C of Fig. S-6 show the original AP and Ca\textsubscript{i}T signals, \(\theta\text{AP}\), \(\theta\text{CaiT}\), and \(\theta\text{AP-CaiT}\) for pixels a, b and c, respectively. One can see that the segments of disorganized relationship between the AP and Ca\textsubscript{i}T (seen in pixels a and c) correspond to segments of \(\theta\text{AP-CaiT}\) signal where both negative (normal) and positive (abnormal) deflections are present in \(\theta\text{AP-CaiT}\). However, similar to the analysis of the phase relationship based on the detection of the upstrokes of the AP and Ca\textsubscript{i}T (see Fig. S-5 above), it is difficult to interpret individual pixel information unless the spatial relationships are investigated. Therefore, we analyzed TSPs of \(\theta\text{AP-CaiT}\). Fig. S-6D shows a TSP of \(\theta\text{AP-CaiT}\) which corresponds to the TSPs
derived from the dual upstroke representation of the same AP and Ca_{i}T movies and shown in Fig.S-5, A-C. Comparing the two representations of the same data, one can see that the areas of abnormal (reversed) $\theta_{\text{AP-CaiT}}$ (orange-red spots in Fig. 6D) are restricted to the sites where the waves are discontinuous in the dual-upstroke TSPs, or the CB sites. Moreover, in general the $\theta_{\text{AP-CaiT}}$ reversal (see Fig. S-6D) is found in the same time-space positions where dual upstroke representation (see Fig. S-5, A-C) reveals unequal (and apparently random) penetration of the respective AP and Ca_{i}T waves into the blocked area (e.g., see the asterisks in Figs. S-5 and S-6).

In summary, $\theta_{\text{AP-CaiT}}$ analysis leads conceptually to the same conclusion as the analysis of dual upstroke representation of the AP and Ca_{i}T movies. Namely, that the sites of apparent Ca_{i}T “disobedience” to the AP are restricted to the immediate vicinity of the CB sites. As we argue in the Discussion of the main manuscript, the exact mechanism and significance of the AP-Ca_{i}T dissociation near the CB sites cannot be fully established at this point. It cannot be excluded that during VF Ca_{i}T waves are able to propagate beyond the sites of conduction block for the AP by a mechanism involving regenerative Ca-induced Ca release from the sarcoplasmic reticulum.\(^6\) On the other hand, it cannot be excluded that the AP-Ca_{i}T dissociation near the CB sites is an artifact of signal averaging through the thickness of the myocardium.\(^7\) In any case, the fact that abolishment of Ca_{i}T by BAPTA-AM does not change WB incidence (see the main text) indicates that the dynamics of Ca_{i}T waves during VF is not essential for VF maintenance in the intact porcine heart.

**Mutual Information of AP and Ca_{i}T signals**

Mutual Information (MI) was used in the past to assess the relationship between the AP and Ca_{i}T during VF.\(^4,8\) Specifically, Omichi et al.\(^4\) reported low value of MI not statistically different from a random relationship. Since we observed a highly non-random relationship everywhere
[Ca$^{2+}$], and wavebreak formation during VF/ Data Supplement

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except at the immediate vicinity of CB sites, we performed a limited analysis of MI mainly to test two hypotheses: 1) MI is heterogeneous over the mapped area depending on the dynamical distribution of CB/WB sites, and 2) the experimentally observed MI is larger than that obtained in random signals. We used $M_{\text{I max}}$ as described in Methods to minimize the influence of the time delay between the two waveforms. Figure S-7A-C shows representative cases of signals with high, intermediate, and low $M_{\text{I max}}$, respectively. A high value of $M_{\text{I max}}$ (0.83, Figure S-7A) was observed in dual AP/Ca$_i$T recordings not affected by the proximity of a SP (no SPs detected in a 5x5-pixel region centered at the pixel in question). Consequently, Ca$_i$T closely follows AP in each activation. Intermediate value of $M_{\text{I max}}$ (0.37, Figure S-7B) was associated with a passage of a SP in the vicinity of the pixel in question. Note that whilst the SP visited only very briefly the central pixel, its influence as it meanders around the neighboring pixels still generates a marked dissociation between AP and Ca$_i$T (Figure S-7B). The spatial pattern of this dissociation is similar to that shown in Figure S-3, such that there is a variable penetration of AP and Ca$_i$T waves into the CB area associated with the meandering SP (not shown). Finally, a low value of $M_{\text{I max}}$ (0.12, Figure S-7C) was usually observed at the very periphery of the mapped area, suggesting that a combination of lower signal-to-noise ratio and enhanced motion artifact could deteriorate both signals leading to an apparent dissociation between the AP and Ca$_i$T. Figure S-7D shows that even though $M_{\text{I max}}$ values were heterogeneous in all experiments, the distributions were similar. Importantly, in each experiment the entire range of $M_{\text{I max}}$ computed after shuffling the AP signals was below even the lowest $M_{\text{I max}}$ values computed for intact AP/Ca$_i$T pairs. This indicates a non-random relationship between the AP and Ca$_i$T everywhere in the mapped area.
Figure Legends

Figure S-1.
Sketch illustrating the topological relationship between CB and WB. A, An instantaneous position of wavefront (WF) before it collides with a refractory wave tail (WT). B, At the very instant of collision between WF and WT, a segment of WF extinguishes because the tissue ahead of WF is refractory. This result in formation of two WBs connected by a line of conduction block (CB). C and D, identification of a CB site using TSP. C, consecutive positions of WF at time instants labeled t1 to t4. D, a TSP along the horizontal pixel line between the two points with coordinates x1,y1 and x2,y1 indicated in C. The time axis in D is downwards. It is seen that the TSP allows to determine the x-coordinate of the CB site at y=y1 with the accuracy of the distance between WF positions detected at t3 and t4. Analysis of TSPs constructed for all horizontal and all vertical pixel lines is sufficient to identify all CB areas in a given movie.

Figure S-2.
Quantification of phase difference between the AP and Ca\textsubscript{i}T (θ\textsubscript{AP-CaiT}) using Hilbert transformation. A, the optical AP signal (top) and its respective phase signal (θ\textsubscript{AP}, bottom). B, the optical Ca\textsubscript{i}T signal (top) and its respective phase signal (θ\textsubscript{CaiT}, bottom). Note that throughout each AP or Ca\textsubscript{i}T cycle, the phase monotonically increases from 0 to 2\pi and then abruptly returns to 0. C, the phase difference between the AP and Ca\textsubscript{i}T (θ\textsubscript{AP-CaiT}) calculated by subtracting θ\textsubscript{CaiT} from θ\textsubscript{AP}. Normally, Ca\textsubscript{i}T cycle is slightly delayed with respect to the AP cycle, leading to sharp negative deflections in θ\textsubscript{AP-CaiT} during the time intervals between the take-off times of the AP and Ca\textsubscript{i}T (t1 and t2, respectively), and a relatively constant, slightly positive value of θ\textsubscript{AP-CaiT} during the rest of the cycle. Examples of abnormal θ\textsubscript{AP-CaiT} dynamics are shown in Fig. S-6.
Figure S-3.
Detailed analysis of AP/Ca,T relationship in the vicinity of pixel (40,26) labeled as site 3 in Figure 3 of the main text. A, The AP and Ca,T recordings from pixel (40,26). Labels (lower case letters) at each deflection in both signals identify a normal AP/Ca,T wave found in the vicinity of pixel (40,26) which caused that particular deflection. B, a sketch of mapped area showing a vertical and horizontal line of pixels centered at pixel (40,26). The AP and Ca,T signals from the vertical pixel line are shown in C and E, respectively. The AP and Ca,T signals from the horizontal pixel line are shown in D and F, respectively. Solid and dashed red lines in C-F show continuous AP or Ca,T waves with amplitude above and below 10% of maximal amplitude, respectively. G, the lines showing the AP (green) and Ca,T (blue) waves are taken from C and E and superimposed for clarity. H, the same is done for lines showing the AP and Ca,T waves in D and F. Labels in C-H are the same as in A and identify normal AP/Ca,T waves in the vicinity of pixel (40,26) which can be traced to each deflection in the AP and Ca,T recordings in that pixel. Subscripts at labels denote a rotation number of reentrant waves.

Figure S-4.
Effect of the amplitude rejection threshold on the AP-Ca,T phase delay distribution. Phase delay was computed as the time difference between the mid-upstrokes of the AP and Ca,T in the same pixel expressed in the number of frames (see Methods in the main manuscript). Top to bottom, histograms of phase delay distribution at three different threshold values (5%, 10%, and 20%, respectively) in a representative experiment.

Figure S-5.
Effect of the amplitude rejection threshold on the spatiotemporal relationship between the AP and Ca,T visualized using TSPs derived from dual wavefront movies. In these movies, the AP wavefronts are coded with green, Ca,T wavefronts are coded with blue, and their overlap is
coded with white. The data is the same as shown in Figs. 3 and 4 of the main text. A-C, dual-wavefront TSPs obtained using 5%, 10%, and 20% rejection threshold, respectively. D and E, expanded view of the area indicated by asterisks in A and B, respectively. F, the AP (green) and Ca,\text{T} (blue) recordings from pixels a, b, and c indicated in the TSPs with red dotted lines. Pixels a, b, and c correspond to pixels located in sites labeled respectively 1-3 in Figure 3 of the main text. For all other notations see Fig. 3 in the main text.

Figure S-6.
Spatiotemporal distribution of $\theta_{AP-CaiT}$ in the same movie as shown in Figure S-5. A-C, original and phase-transformed AP and Ca,T signals from pixels a to c, respectively. Top, the original AP (green) and Ca,T (blue) signals. Middle, $\theta_{AP}$ (blue) and $\theta_{CaT}$ (green). Bottom, $\theta_{AP-CaiT}$. Arrows indicate instances of reversed $\theta_{AP-CaiT}$. D, a TSP of $\theta_{AP-CaiT}$ which corresponds to the same line of pixels and time interval as dual-wavefront TSPs shown in Fig. S-5, A-C. In this TSP, the range of $\theta_{AP-CaiT}$ is color-coded using a palette from magenta (-2$\pi$) to red (+2$\pi$). Thus, the narrow magenta-blue bands represent normal sequences of AP-CaiT upstrokes (Ca,T upstroke lagging behind the AP upstroke), whereas orange-red spots represent reversed sequences (Ca,T upstroke is ahead of the AP upstroke). Asterisks are in the same positions as in Fig. S-5 and denote a CB site. Note that the occurrences of reversed $\theta_{AP-CaiT}$ (orange-red spots) are found exclusively in the vicinity of CB sites.

Figure S-7.
Analysis of Ml_{max} between AP and Ca,T. A-C, Sample dual recordings from pixels with high, intermediate, and low Ml_{max}, respectively. In A and B, the three solid lines underneath signal recordings indicate the presence (step) or absence (line) of an SP detected in the pixel itself (top line), first 8 neighboring pixels (middle line), and second 16 neighboring pixels (bottom line). D, Ml_{max} statistics measured in all pixels in 7 experiments. Bars show the range (minimum to
maximum); dots and error bars show the mean and the standard deviation, respectively. Upper bars represent $M_{\text{Imax}}$ measurements in intact signals. Lower bars represent $M_{\text{Imax}}$ measurements after AP signals were shuffled.

**Supplemental videos**

**Movie 1.** Dual AP and $\text{Ca}_tT$ movies before (control) and after BAPTA-AM. 1. AP movie during control. 2. $\text{Ca}_tT$ movie during control. 3. AP movie after BAPTA-AM. 4. $\text{Ca}_tT$ movie after BAPTA-AM. Playback speed reduced (x60).

**Movie 2.** AP phase movie in the absence of BAPTA-AM. Playback speed reduced (x60).

**Movie 3.** AP phase movie in the presence of BAPTA-AM. Playback speed reduced (x60).

**References**


Figure S-1
Figure S-2
Figure S-3
Figure S-4
Figure S-5
Figure S-6

A  pixel a

B  pixel b

C  pixel c

D  Image with color bar indicating 250 ms and 5 mm scales.

\[ 0 \leq x, y \leq \pi \]
Figure S-7

A

\[ MI_{\text{max}} = 0.83 \]

B

\[ MI_{\text{max}} = 0.37 \]

C

\[ MI_{\text{max}} = 0.12 \]

D

Experiment

\[ 0.0 \quad 0.2 \quad 0.4 \quad 0.6 \quad 0.8 \quad 1.0 \]

Figure S-7