Defects in T-tubular electrical activity underlie local alterations of calcium release in heart failure

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Action potentials (APs), via the transverse axial tubular system (TATS), synchronously trigger uniform Ca2+ release throughout the cardiomyocyte. In heart failure (HF), TATS structural remodeling occurs, leading to asynchronous Ca2+ release across the myocyte and contributing to contractile dysfunction. In cardiomyocytes from failing rat hearts, we previously documented the presence of TATS elements which failed to propagate AP and displayed spontaneous electrical activity; the consequence for Ca2+ release remained, however, unsolved. Here, we develop an imaging method to simultaneously assess TATS electrical activity and local Ca2+ release. In HF cardiomyocytes, sites where T-tubes fail to conduct AP show a slower and reduced local Ca2+ transient compared with regions with electrically coupled elements. It is concluded that TATS electrical remodeling is a major determinant of altered kinetics, amplitude, and homogeneity of Ca2+ release in HF. Moreover, spontaneous depolarization events occurring in failing T-tubules can trigger local Ca2+ release, resulting in Ca2+ sparks. The occurrence of tubule-driven depolarizations and Ca2+ sparks may contribute to the arrhythmic burden in heart failure.

Significance

The plasma membrane of cardiac myocytes contains complex invaginations known as transverse tubules (T-tubules). In heart failure, T-tubule loss is a major contributor to Ca2+ transient abnormalities, leading to weaker and slower contraction. Current therapeutic strategies are often based on attempts to accelerate Ca2+ transients. Here, we demonstrate that T-tubular loss represents just one way by which T-tubule dysfunction leads to asynchronous Ca2+ release across the myocyte. In fact, we report that defects in T-tubular electrical activity may contribute to Ca2+ mediated arrhythmogenesis not only by favoring asynchronous Ca2+ release, but also by generating voltage-associated Ca2+ sparks. This work provides the first description to our knowledge of these novel proarrhythmic events that could help guide future therapeutic strategies.

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and homogeneous labeling of the sarcolemma by the VSD (magenta), whereas FluoForte (green) is present in the whole cell. The RAMP microscope is used to probe both AP and [Ca\(^{2+}\)] at different sites by scanning multiple noncontiguous sarcolemmal regions with the excitation beam. Fig. 1C is an example of real-time simultaneous optical recording from six different membrane areas (white lines in Fig. 1B) located at the surface sarcolemma (SS) and in five T-tubules (TTi). AP is elicited at 200 ms (black arrowheads). Membrane voltage (magenta) and [Ca\(^{2+}\)] (green).

To study the Ca\(^{2+}\) transient kinetics, the activity of each site is followed during steady-state stimulations (Fig. 2A), in the absence (CTRL) and presence of the \(\beta\)-adrenergic agonist isoproterenol (ISO, 10\(^{-5}\) M). Ca\(^{2+}\) transients measured in CTRL within cytoplasmic regions close to SS and TTs perfectly overlap with no significant variation in time-to-peak (TTP in Fig. 2B) and 50\% decay (Ca\(^{2+}\)T\(_{50}\) in Fig. 2B). This result demonstrates the uniformity of Ca\(^{2+}\) release across the whole cell. As expected, ISO significantly speeds Ca\(^{2+}\) transients, reducing TTP and Ca\(^{2+}\)T\(_{50}\) both in SS and TTs (Fig. 2B). Further, the traces show a nonnegligible beat-to-beat variability, which we estimate by the coefficient of variation (CV, defined as the ratio of the SD to the mean); in Fig. 2C we report the CV of both Ca\(^{2+}\) transients kinetics and amplitude (AMP). The nonratiometric nature of our Ca\(^{2+}\) indicator precludes assessing the absolute value of Ca\(^{2+}\) transient amplitude; however, within the same cell, the CV of amplitude is informative. Isoproterenol significantly reduces the variability of both amplitude and kinetics of Ca\(^{2+}\) transients. These observations suggest that we are looking at the stochastic nature of Ca\(^{2+}\) release. Indeed, only a few Ca\(^{2+}\) release units (CRUs) reside within the volume probed by each scanned line (5–10 CRUs in \(~10\) \(\mu\)m\(^3\)) (11, 12). The recruitment of CRUs probed on each scanned line may vary stochastically, leading to beat-to-beat changes of the recorded transients. Isoproterenol increases the open probability of the CRUs, enhancing Ca\(^{2+}\) transients synchronicity.

The ability of our method to probe the spatiotemporal relationship between Ca\(^{2+}\) and electrical activity is then explored in a model of acute detubulation. Using a formamide-based osmotic-shock technique, it is possible to physically disconnect tubules from the SS (10, 13). Disconnecting TTs would prevent the diffusion of the dye into TATS. However, if staining is performed before detubulation, even SS-disconnected TTs will maintain their labeling (Fig. 3A). As previously demonstrated (10), in this condition we find that the elicited AP is clearly visible in the SS, but it is absent in (74 \pm 6\%) of TTs. Fig. 3B depicts three representative traces (average of 10 trials) from the cell shown in Fig. 3A (white lines). SS, as well as TT, exhibits a proper AP and Ca\(^{2+}\) transient; TT, on the other hand, fails to propagate AP, indicating it underwent the formamide-induced disconnection. Our method of simultaneous AP and Ca\(^{2+}\) recording highlights an interesting future of disconnecting tubules: TT shows a remarkably delayed Ca\(^{2+}\) transient. Superimposing alternatively TT and SS Ca\(^{2+}\) traces, it is clear that TT Ca\(^{2+}\) transient is comparable to that at SS, whereas TT Ca\(^{2+}\) transient is delayed (Fig. 3C). A close-up of the TT and SS superimposition shows that such delay mainly affects Ca\(^{2+}\) rise, suggesting that Ca\(^{2+}\) release in the junctional SR adjacent to TT is the result of Ca\(^{2+}\) propagation from the rest of the cell and not of the direct activation of local CRUs. Discriminating the two populations of tubules, i.e., electrically coupled (AP+) and uncoupled TTs (AP−), we find that AP− tubules exhibit a significantly delayed TTP and Ca\(^{2+}\)T\(_{50}\) of Ca\(^{2+}\) transient compared with both AP+ and CTRL tubules (Fig. 3D). In addition, we observe that coupled TTs display bigger Ca\(^{2+}\) amplitude than uncoupled ones (AMP\(_{AP^+}\)/AMP\(_{AP^-}\) = 1.9 \pm 0.7). These results

Fig. 1. Simultaneous multisite voltage and Ca\(^{2+}\) recording. (A) Scheme of the random access multiphoton (RAMP) microscope. It consists of a 1064-nm fiber laser, an acousto-optic modulator (AOM) for angular-spreading precompensation, and two orthogonally mounted acousto-optic deflectors (AODs) (AOD-x and AOD-y) for laser scanning. The fluorescence signal is collected in forward and backward directions using four independent photomultipliers (PMTs), two for the voltage and two for the calcium signals. (Inset) The emission spectra of the Ca\(^{2+}\) probe (dark gray) and VSD (light gray) together with the band-pass filter used for each channel. (B) Two-photon fluorescence (TPF) image of a stained rat ventricular myocyte: sarcolemma in magenta (di-4-ANE(F)PTEA) and [Ca\(^{2+}\)] in green (GFP-certified FluoForte). (Scale bar, 5 \(\mu\)m). (C) Normalized fluorescence traces (ΔF/ΔF\(_{0}\)) simultaneously recorded from the scanned sites indicated in white in B: surface sarcolemma (SS) and five T-tubules (TTi). AP is elicited at 200 ms (black arrowheads). Membrane voltage (magenta) and [Ca\(^{2+}\)] (green).

Fig. 2. Stochastic nature of Ca\(^{2+}\) release. (A) Ten subsequent [Ca\(^{2+}\)] fluorescence traces (ΔF/ΔF\(_{0}\)) close to SS and close to a TT of a control (CTRL) and an isoproterenol-treated (ISO) rat cardiomyocyte. Isoproterenol is used at 10\(^{-5}\)M. AP is elicited at 200 ms (black arrowhead). (B) Graphs showing mean values for Ca\(^{2+}\) transient time-to-peak (TTP) and 50\% of Ca\(^{2+}\) decay (Ca\(^{2+}\)T\(_{50}\)). (C) Graph showing the variability (coefficient of variation, CV) of Ca\(^{2+}\) release amplitude (AMP), TTP, and Ca\(^{2+}\)T\(_{50}\) in the same sites at subsequent stimulated events. Each bar represents the mean \pm SE. Data from 27 SS, 124 TTs (27 CTRL cells) and 11 SS, 48 TT (11 ISO cells). Asterisks indicate significant differences (Student t test, **P < 0.05, ***P < 0.01, ****P < 0.001).
clearly show how the single-tubule activity defines the kinetics and amplitude of local Ca²⁺ transients.

The pathological implications of these findings are then investigated in failing hearts (Fig. S2). We previously observed that TATS structural remodeling occurring in a rat model of postischemic HF (5) is associated with abnormal electrical activity in several tubular elements (10). Here, we investigate how alterations of electrical function of TTs determine anomalies of local Ca²⁺ dynamics in HF (Fig. 4A). We find that in HF, (6.3 ± 1.3)% of TTs fail to propagate AP. Fig. 4B shows a representative measurement in which SS and TT₁ exhibit proper APs, whereas TT₂ displays AP failure associated with a delayed Ca²⁺ transient. In line with previous results (10), tubules showing a two-state scenario characterized by regular APs (AP⁺) and failure events (AP⁻) on a beat-to-beat basis are also observed in HF cells. Distinguishing AP⁻ and AP⁺, we observe that AP⁻ sites display significantly delayed Ca²⁺ transients compared with AP⁺ and to CTRL TT. We also observe a delay of CaTₚ₀ in AP⁺ and AP⁻ sites compared with CTRL TTs (Fig. 4C) as well as a reduction of Ca²⁺ amplitude in AP⁻ versus AP⁺ TATS regions (AMPₚ₀/AMP⁺ = 1.6 ± 0.2). These findings show that electrically uncoupled tubules produce local anomalies of Ca²⁺ release, which cannot be overridden by neighboring functional tubular elements and which contribute to determine inhomogeneous Ca²⁺ cycling. At variance with the acute detubulation model, HF also displays a significant increase of Ca²⁺ TTP nearby SS and AP⁺ compared with CTRL. Although HF is a composite of multiple pathological events (14–23), our findings highlight the critical role of electrically failing TATS in this disease.

The sensitivity of our method is sufficient to also detect the presence of local spontaneous Ca²⁺ events (Fig. 5A), i.e., Ca²⁺ sparks (24, 25). Ca²⁺ sparks are observed at any time during the Ca²⁺ cycle, either in systole or in diastole. In CTRL cells, a detectable membrane potential variation is never observed corresponding with the spark occurrence. The S/N is increased, aligning and averaging 47 spark events; the spark occurrence time (blue arrow in Fig. 5B) is used to align the corresponding voltage traces. Even in this case, we find that Ca²⁺ sparks are not associated with any detectable local membrane potential variations above noise (~5 mV). This result is in full agreement with our previous findings that demonstrated the voltage space-clamp of TATS (10). Although spark-mediated Ca²⁺ rise can generate inward currents due to activation of Na⁺/Ca²⁺ exchanger (26, 27), the high cell capacitance guaranteed by the tight electrical coupling of TATS prevents membrane voltage variations. The frequency of Ca²⁺ sparks (sp) detected in CTRL cells is then compared with those found in acute and chronic detubulation models (Fig. 5C). Electrically coupled tubules in acute detubulation show a spark frequency not significantly different from CTRL, whereas failing tubules (AP⁻) exhibit a significant increase. Because the amplitude of Ca²⁺ release adjacent to AP⁻ tubules is decreased (see above), Ca²⁺ may locally accumulate in the junctional SR, increasing RyR2 open probability (28–30) and, thus, spark rate. A similar trend also holds true for HF. Here, however, even AP⁺ show a spark frequency significantly higher than CTRL. These results suggest that the local control of Ca²⁺ release is profoundly affected by several signaling pathways, and Ca²⁺ accumulation plays a contributory role in spontaneous Ca²⁺ leakage.

Finally, (40 ± 10)% of HF AP⁻ tubules display spontaneous electrical activity, in agreement with our previous work (10). In Fig. 6A we report examples of abnormal electrical events recorded from HF myocytes without or with isoproterenol application (HF+ISO). These spontaneous depolarizations (SD) do not propagate to the neighboring sarcomedial sites and may display a quite heterogeneous shape. Indeed, they can be as fast as an AP or manifest a slower depolarization rise (Fig. 6A). Abnormal spontaneous depolarizations are never observed in CTRL or acutely detubulated cells. The exclusive presence of SD in HF AP⁻ tubules suggests a specific pathology-mediated functional remodeling occurring at the failing sites. Moreover, the frequency of spontaneous electrical events does not depend on β-adrenergic stimulation (Fig. 6B). Interestingly, (15 ± 10)% of spontaneous depolarization events are associated with a corresponding local

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**Fig. 3.** Delay of Ca²⁺ release in AP-failing TT of acute detubulated cells. (A) TPF image of a stained rat ventricular myocyte after formamide-induced osmotic shock; membrane in magenta and [Ca²⁺]; in green. (Scale bar, 5 μm.) (B) Average of 10 subsequent fluorescence traces (ΔF/F₀) of voltage (magenta) and [Ca²⁺]; (green) recorded at the three different sites indicated in A: SS and two TT. APs are elicited at 200 ms (black arrowheads). (C) Superposition of the Ca²⁺ traces nearby SS and TT (Top) and of SS and TT (Middle). (Bottom) A close-up of TT₂ and SS superposition (interval indicated by dashed line in the middle trace). (D) Graphs showing mean values for Ca²⁺ release TTP and CaTₚ₀ discriminating electrically coupled (AP⁺) and uncoupled (AP⁻) tubules. Ochre lines represent the Ca²⁺ kinetics features measured nearby TT of CTRL: mean (solid) ± SE (dashed). Asterisks indicate significant differences (Student t test, *P < 0.05, **P < 0.01, ***P < 0.001). Ochre asterisks refer to the comparison with CTRL values. Data from 8 SS, 14 AP⁺ TTs, and 41 AP⁻ TTs (nine cells).

**Fig. 4.** Ca²⁺ release in AP-failing TT of HF. (A) TPF image of a stained rat ventricular myocyte isolated from a failing heart; membrane in magenta and [Ca²⁺]; in green. (Scale bar, 5 μm.) (B) Average of 10 subsequent fluorescence traces (ΔF/F₀) from the scanned lines indicated in A: SS and TT. APs are elicited at 200 ms (black arrowheads). Membrane voltage in magenta and [Ca²⁺]; in green. The gray dashed line indicates the Ca²⁺ release time-to-peak measured nearby SS, shown for comparison. (C) Graphs showing Ca²⁺ release TTP and CaTₚ₀ nearby SS and TT. The failing TTs (AP⁻) have been distinguished from the electrically responsive ones (AP⁺). Ochre lines represent the Ca²⁺ kinetics features measured nearby TT of CTRL: mean (solid) ± SE (dashed). Asterisks indicate significant differences (Student t test, *P < 0.05, **P < 0.01, ***P < 0.001). Ochre asterisks refer to the comparison with CTRL values. Data from 59 SS, 364 AP⁺ TTs, and 23 AP⁻ TTs (59 HF cells).
Ca$^{2+}$ spark, which we call voltage-associated Ca$^{2+}$ spark (V-spark; Fig. 5A). In our experimental conditions, V-sparks do not propagate but remain confined in the probed sites where they arise (Fig. S3) and are always detected 15–50 ms after the local spontaneous electrical activity. On the other hand, we have never detected a membrane-potential variation as a consequence of a spontaneous Ca$^{2+}$ spark, contradicting the hypothesis that spontaneous TT activity results from local Ca$^{2+}$ release from the SR. The percentage of spontaneous depolarization events associated with a corresponding Ca$^{2+}$ spark is not affected by isoproterenol treatment (Fig. 6D).

**Discussion**

The synchronization of Ca$^{2+}$ release within ventricular cardiomyocytes is ensured by AP propagation across TATS. Here, an optical method is used to dissect the spatiotemporal relationship between TATS electrical activity and Ca$^{2+}$ release in healthy and diseased cells. In an acute detubulation model, tubules failing to propagate AP show a delayed Ca$^{2+}$ release, as well as a reduction in Ca$^{2+}$ transient amplitude. Failing tubules are unable to activate Ca$^{2+}$ channels; Ca$^{2+}$ release is probably triggered by Ca$^{2+}$ propagating from neighboring activated junctional sites. This result highlights how the electrical activity of a single TATS element is crucial in determining the local Ca$^{2+}$ release, even where an intact excitation–contraction coupling machinery is present.

In HF, altered intracellular Ca$^{2+}$ handling is a major culprit of impaired force generation and relaxation, thereby causing systolic and/or diastolic dysfunction (31). T-tubular loss was considered a main contributor to these Ca$^{2+}$ transients abnormalities (7), resulting in a weaker and slower contraction (5, 32). Here we find a global delay of Ca$^{2+}$ transient occurring even close to electrically coupled TTs. It might be ascribed to several pathological factors such as an altered dyad microarchitecture (8, 33). In addition, we give evidence that the presence of failing APs in HF locally impairs Ca$^{2+}$ transients. This Ca$^{2+}$ alteration leads to nonuniform myofilament activation, promoting proarrhythmogenic electromechanical feedback (34). We confirm that a number of tubules display a two-stage scenario characterized by regular APs and failure events (10), affecting Ca$^{2+}$ amplitude and kinetics on a beat-to-beat basis. In HF such variability is five times larger than that found in CTRL. This phenomenon represents an SR-independent mechanism that generates local Ca$^{2+}$ alternans (28, 35, 36). Both nonuniform myofilament activation and local Ca$^{2+}$ alternans represent fundamental mechanisms of contractile dysfunction and arrhythmias. HF of any cause is always characterized by an increased risk of arrhythmias, resulting from a number of changes at the tissue level (e.g., myocardial fibrosis, scars, altered cell-to-cell contact) causing a higher propensity for reentry. However, to initiate a sustained arrhythmia, a premature conducted depolarization is needed, and those arrhythmic triggers always stem from the anomalous electrical function of a single cardiomyocyte. Defects in T-tubular electrical activity found in HF may contribute to Ca$^{2+}$-mediated arrhythmogenesis not only by favoring asynchronous Ca$^{2+}$ release and alternans, but also by generating voltage-associated Ca$^{2+}$ sparks. In fact, ~15–20% of the spontaneous depolarizations in remodeled TATS are followed by a local Ca$^{2+}$ release, resembling a Ca$^{2+}$ spark; we named this category of calcium release events “V-spark”. A V-spark arises when spontaneous voltage depolarizations are large enough to activate Ca$^{2+}$ channels, leading to Ca$^{2+}$ entry via DHPR. This local Ca$^{2+}$ influx triggers Ca$^{2+}$ release from the SR, generating a V-spark. In that scenario, the depolarizing Na$^+$/Ca$^{2+}$ exchange current ($I_{\text{NaX}}$) may not play

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**Fig. 5.** Ca$^{2+}$ sparks in failing tubules. (A) Representative fluorescence traces ($\Delta F/F_0$) from two CTRL rat cardiomyocytes. The blue arrows highlight the presence of spontaneous Ca$^{2+}$ sparks. AP is elicited at 200 ms (black arrowheads). Membrane voltage (magenta) and [Ca$^{2+}$], (green). (B) The trace shows the corresponding membrane potential value of 47 aligned and averaged CTRL Ca$^{2+}$ sparks. The blue arrow shows the alignment point. (C) Graph showing the frequency of Ca$^{2+}$ sparks (s) occurring in AP+ and AP−tubules of acutely detubulated and HF cells. Each bar represents the mean ± SE. Ochre lines represent the Ca$^{2+}$ spark frequency occurring nearby TTs of CTRL: mean (solid) ± SE (dashed). Asterisks indicate significant differences (Student t test, *P < 0.05, ***P < 0.001). Ochre asterisks refer to the comparison with CTRL values.

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**Fig. 6.** Voltage-associated Ca$^{2+}$ sparks (V-sparks). (A) Fluorescence traces ($\Delta F/F_0$) from two failing TTs from HF cardiomyocytes and two failing TTs from isoproterenol-treated HF cells (HF+ISO) displaying spontaneous electrical activity. Electrical trigger at 200 ms (black arrowheads). Membrane voltage in magenta and [Ca$^{2+}$], in green. (B) Graph showing the frequency of spontaneous depolarization events ($\Delta F/F_0$) in AP+ and AP−tubules of HF and HF+ISOC cells. Each bar represents the mean ± SE. (C) Fluorescence traces ($\Delta F/F_0$) of two failing TTs, one from a HF and one from a HF+ISO displaying voltage-associated Ca$^{2+}$ sparks. Bottom: Close-ups of the correspondent graphs above (dashed lines interval). Voltage and Ca$^{2+}$ traces have been differently magnified (y axis) to better compare the occurrence timing of the spontaneous events. (D) Graph showing the percentage of spontaneous depolarization events that are associated with a correspondent local Ca$^{2+}$ spark (V-sparks). Each bar represents the mean ± SE.
a crucial role, because Ca\textsuperscript{2+} release always precedes, rather than follows, spontaneous depolarization. V-spark frequency is not modified by the application of isoproterenol. This suggests that, in a pathological setting, such as HF, triggering events may not depend on alterations of RyR2 channel function [e.g., in response to its phosphorylation levels by protein kinase A or Ca\textsuperscript{2+} calmodulin Kinase II (37)]; rather, they appear to be a direct consequence of the anomalous local membrane activity. Our experimental conditions featuring low inotropic levels, to minimize cell movement and to isolate single events, did not allow propagation of cell-spanning Ca\textsuperscript{2+} waves arising from V-sparks. Although we could not directly demonstrate the arrhythmogenic potential of V-sparks, they are likely to play a significant role in increasing the rate of nontriggered Ca\textsuperscript{2+} release events in HF. Most interestingly, these events appear to be insensitive to β-adrenergic signaling, a well-established arrhythmogenic stimulus. Thus, it is tempting to speculate that incomplete protection against life-threatening arrhythmias by beta-blockade (38) might be due to enduring arrhythmogenic mechanisms in HF mediated by V-sparks, whose fine molecular machinery remains to be clarified.

In conclusion, our results show that functional defects of T-tubules occurring in HF significantly contribute to the pathophysiology of this disease and need to be addressed by future therapeutic strategies aiming to reduce contractile dysfunction and arrhythmias in patients.

Methods

Cardiomyocyte Preparations and Labeling. Ventricular myocytes were isolated from male Wistar Han rats (300–350 g, Harlan Laboratories, SRL), as previously described (10). Acute detubulation was induced by osmotic shock (13). Briefly, 1.5 M formamide was added to the cell suspension for 15–20 min; the cells were then rapidly resuspended in standard, formamide-free solution. Mechanical infarction is induced by ligation of the left anterior coronary artery, as previously described (5). In this class of experiments, male Wistar Han rats (190–230 g, Harlan Laboratories, SRL) were used. Cardiac function was monitored with echocardiography before surgery and was periodically checked after the intervention. Six weeks after the infarction, a left ventricular dilatation occurs, together with a loss of contractile function (Fig. S2). Rats were killed 6-8 wk after surgery and used for cell isolation. All of the experiments were designed in accordance with the rules of the Italian Ministry of Health. Cells were loaded in extracellular buffer added with 10 μM blebbistatin, 4 μM cytochalasin D, and 500 μM CaCl\textsubscript{2}. First, 0.5 μg/mL of GFP-certified Fluoforte dissolved in DMSO were added to the cell suspension for 15 min. After washing, 2 μg/mL of di-4-AN(F)EPPTEA dissolved in ethanol was also added for 15 min, and then cells were resuspended in fresh extracellular buffer containing 10 μM blebbistatin, 4 μM cytochalasin D, and 1 mM CaCl\textsubscript{2}. Loaded preparations were used for experiments within 1 h. The staining and imaging sessions were performed at room temperature (20 °C). The di-4-AN(F)EPPTEA dye shows a sensitivity 18%/100 mV (10) with a submillisecond time response (9) whereas the GFP-certified Fluoforte is characterized by a K\textsubscript{D} in the range of 380–400 nM and quantum yield (Ca\textsuperscript{2+}) bound of 0.18.

The RAMP microscope and optical recording. The basic design of our RAMP imaging system has already been described (10). Now, however, the system was supplemented with a two-color detection scheme (Fig. 1A). The fluorescence signal was collected in backward direction (BWD) by the excitation oil immersion objective (63× numerical aperture 1.4; Zeiss) and in forward direction (FWD) using a high numerical aperture condenser lens. For each detection direction, a dichroic mirror was used to split the two spectral components of the fluorescence signal, the red and the emission light. The fluorescence signal was detected by two independent photon-counting modules based on the GaAsP photomultiplier tube (PMT) (H7422, Hamamatsu). Emission filters of 655 ± 20 and 520 ± 16 nm were used for voltage and Ca\textsuperscript{2+} detection, respectively. For each color, the PMT current pulses coming from the two independent PMTs were summed, integrated, and digitized to obtain an intensity fluorescence map as a function of the beam position. The measurements were performed during steady-state stimulation (0.34 Hz). The cells were field-stimulated using two parallel platinum wires (250 μm in diameter) placed at a distance of 6.3 mm. Square pulses of 10–20 V and duration of 3 ms were used to reach AP threshold. In a typical measurement, we probed 5–10 different sarcomemal sites for 10 subsequent trials. The length of the scanned lines ranged from 2 to 10 μm with an integration time per membrane pass of ~200 μs, leading to a temporal resolution of 0.4–2 ms. As shown in Fig. 1A, the large Stokes shift of fluorinated VSD is not sufficient to prevent spectral contamination between the two channels. For this reason, we optimized a simple unmixing procedure under two hypotheses: negligible contamination of the green channel on the red one and constant VSD sensitivity across the emission spectrum. The contamination between the channels was quantified to remove the proper fraction of the red signal on the green channel (Fig. S1).

Data Analysis. Optical data were analyzed with software written in LabVIEW 2010 (National Instruments). The amplitude and kinetics parameters of the Ca\textsuperscript{2+} were manually identified for a trace by trace for the calculation of the co-efficient of variation (CV), whereas the mean values of each probed site were determined after averaging 10 subsequent trials to increase accuracy. Spontaneous Ca\textsuperscript{2+} sparks were scored when a sudden increase of fluorescence intensity occurred with a ΔF/F\textsubscript{0} twofold above the trace noise not correlated to the electrical stimulus. VSD sensitivity was estimated based on the evidence that AP amplitude of 100 mV corresponds to a fluorescence variation of 20%. The T-tubules showing failures were scored using threshold ΔF/F\textsubscript{0} = 0.037 in agreement with our previous findings (10). Spontaneous electrical activity was scored when a sudden increase of fluorescence intensity occurred with a ΔF/F\textsubscript{0} > 0.074 not correlated to the stimulus.

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