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Rapid communication

Calcium waves driven by "sensitization" wave-fronts

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Abstract

Objective: Cellular Ca^{2+} waves are understood as reaction–diffusion systems sustained by Ca^{2+}-induced Ca^{2+} release (CICR) from Ca^{2+} stores. Given the recently discovered sensitization of Ca^{2+} release channels (ryanodine receptors; RyRs) of the sarcoplasmic reticulum (SR) by luminal SR Ca^{2+}, waves could also be driven by RyR sensitization, mediated by SR overloading via Ca^{2+} pump (SERCA), acting in tandem with CICR.
Methods: Confocal imaging of the Ca^{2+} indicator fluo-3 was combined with UV-flash photolysis of caged compounds and the whole-cell configuration of the patch clamp technique to carry out these experiments in isolated guinea pig ventricular cardiomyocytes.
Results: Upon sudden slowing of the SERCA in cardiomyocytes with a photoreleased inhibitor, waves indeed decelerated immediately. No secondary changes of Ca^{2+} signaling or SR Ca^{2+} content due to SERCA inhibition were observed in the short time-frame of these experiments.
Conclusions: Our findings are consistent with Ca^{2+} loading resulting in a zone of RyR 'sensitization' traveling within the SR, but inconsistent with CICR as the predominant mechanism driving the Ca^{2+} waves. This alternative mode of RyR activation is essential to fully conceptualize cardiac arrhythmias triggered by spontaneous Ca^{2+} release.

Keywords: Calcium; SR; Ca^{2+} pump; Myocytes; Ec-coupling

1. Introduction

Intracellular Ca^{2+} signals are encoded in various ways, such as their amplitude, frequency and subcellular spatial localization, which allows the targeting of specific cellular reactions with the universal second messenger Ca^{2+}. In cardiac muscle, the amplitude of cellular Ca^{2+} signals is locally controlled by Ca^{2+} influx via L-type Ca^{2+} channels and subsequent amplification by CICR from the SR. Since this amplification system exhibits positive feedback, it has an inherent tendency to oscillate, particularly under pathological conditions such as SR Ca^{2+} overload. These oscillations are clinically important as they are known to trigger arrhythmias [1]. On the cellular level, Ca^{2+} oscillations often manifest themselves as Ca^{2+} waves [2,3]. Propagation of Ca^{2+} waves in both excitable and unexcitable cells is thought to be sustained by CICR [4–6]. However, in cardiac muscle this paradigm faces the conceptual complexity of having to reconcile the low Ca^{2+} sensitivity [7] of the Ca^{2+}-release channels (~50 to 100 μM for the ryanodine receptor (RyR)) [8,9] with the fact that the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) at the wave-front rarely exceeds 1 μM [2,10]. However, recent observations on the termination of Ca^{2+} release suggest a prominent role for the luminal intra-store Ca^{2+} concentration ($[Ca^{2+}]_{SR}$) in sensitizing the RyRs towards Ca^{2+} triggers from the cytosolic side of the channel [11]. Such a mechanism suggests that Ca^{2+} waves could, in principle, be mainly driven by a region of RyR sensitization traveling *inside* the sarcoplasmic reticulum at the wave-front, with the sensitization being mediated by local SR Ca^{2+} (over) loading via the SERCA.

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2. Methods

2.1. Cell isolation

Experiments were carried out according to the Swiss Animal Protection Law and conform with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Ventricular myocytes were isolated from male guinea-pig hearts using established enzymatic methods based on collagenase and protease digestion [12]. Hearts were rapidly excised after cervical dislocation and mounted on a Langendorff apparatus for retrograde perfusion with nominally Ca^{2+} free solution followed by enzyme containing solution for 5 min each.

2.2. Current recordings and solutions

Cardiomyocytes were voltage clamped in the whole-cell configuration of the patch-clamp technique. Internal solution contained (mM): Cs-Asp 120, HEPES 20, TEA-Cl 20, K-ATP 5, NaCl 8, *O*-[*o*-nitromandelyloxycarbonyl]-2,5-di (*tert*-butyl)hydroquinone (Nmoc-DBHQ) 1, fluo-3-K_5 0.05,

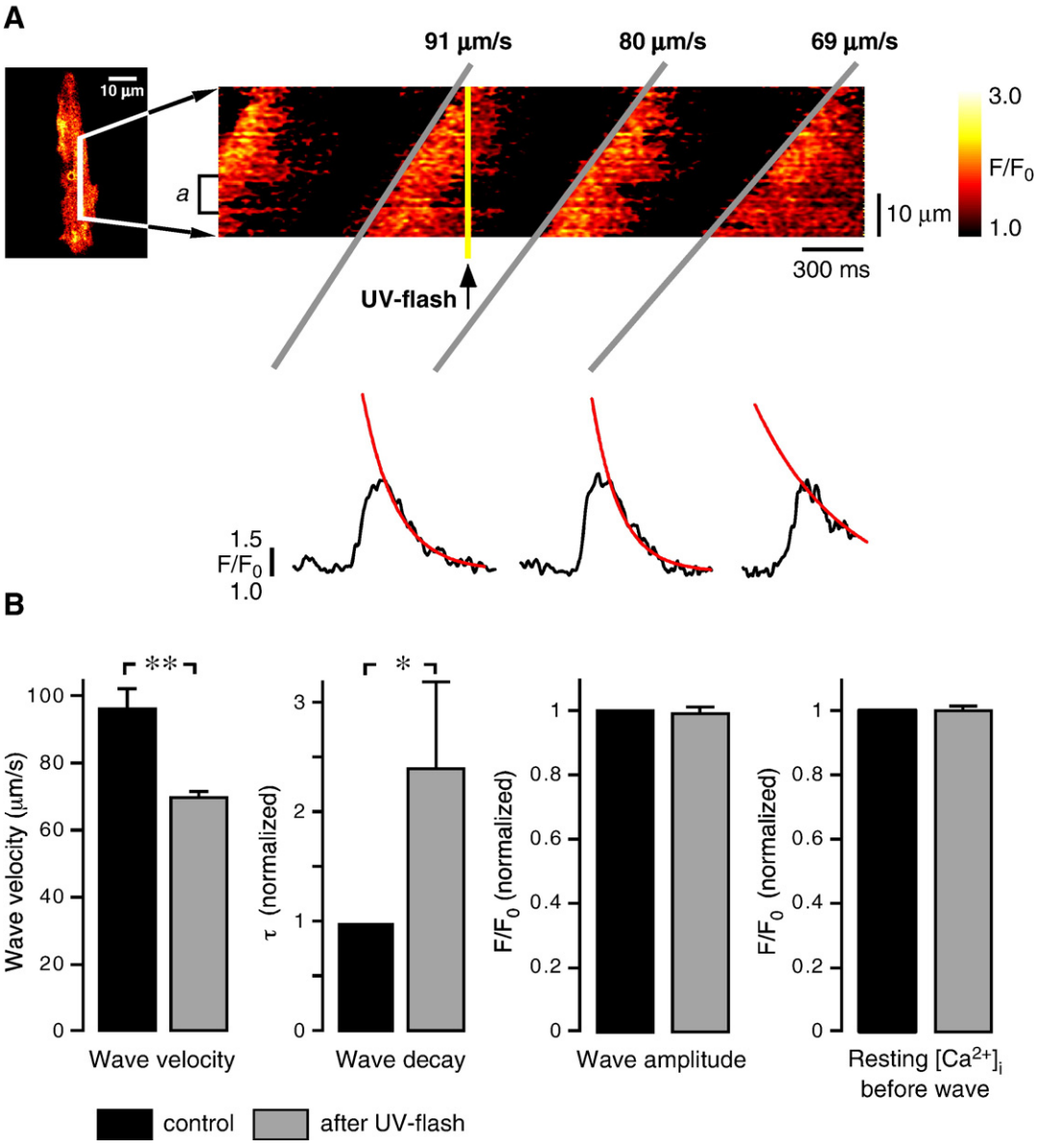

Fig. 1. Photorelease of the SERCA inhibitor DBHQ affects Ca^{2+} wave propagation velocity. (A) Cell image and example of a confocal line-scan recording with photorelease of DBHQ during spontaneous Ca^{2+} waves (provoked by 6 mM $[Ca^{2+}]_o$). Angled gray lines mark the wave-fronts (91 µm/s before, 80 µm/s and 69 µm/s after flash). The trace was extracted from the line-scan image (a) by averaging after aligning the lines. Red lines show monoexponential fits to the wave decay. (B) Statistical analysis in control and after photorelease of DBHQ (maximal effect observed within 1 s): wave velocity 95.8±5.9 µm/s was slowed to 69.3±1.8 µm/s. No alterations in wave amplitude (0.99±0.02 of control) or in resting $[Ca^{2+}]_i$ (0.99±0.01 of control) were observed after the UV-flash, confirming absence of photolytic side-effects. Consistent with the inhibition of the SERCA, the τ of wave-decay was longer after a UV-flash (2.39±0.78 of control). (*: $p<0.05$; **: $p<0.01$, $n=5$ cells).

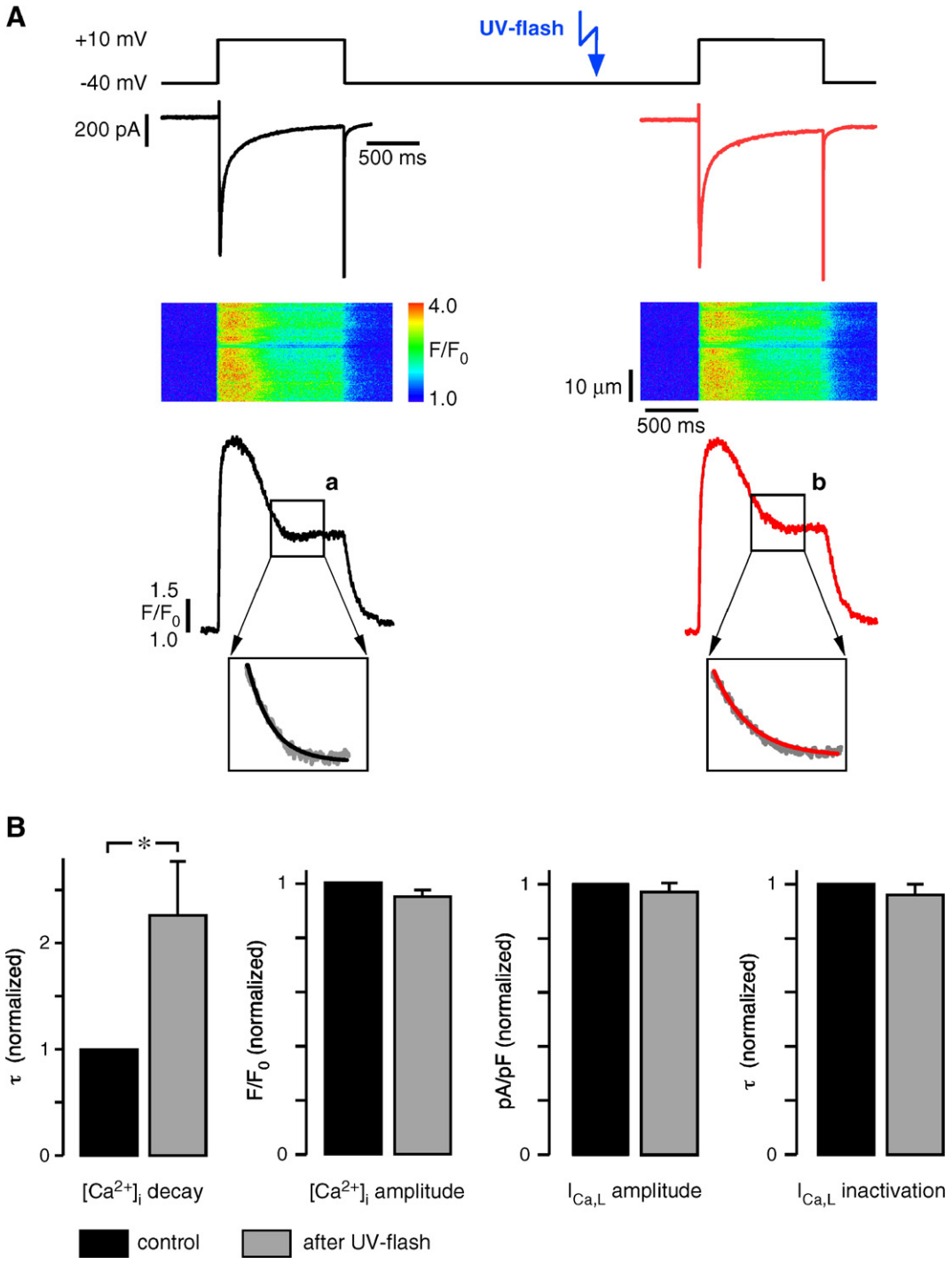

Fig. 2. During Ca^{2+} transients elicited by $I_{Ca,L}$ only the Ca^{2+} removal is slowed by photorelease of DBHQ, $I_{Ca,L}$ and CICR were unaffected. (A) $I_{Ca,L}$ before (left) and after UV-flash (right) and the corresponding Ca^{2+} transients in 1.8 mM extracellular [Ca^{2+}]. Monoexponential function fitted to decay of Ca^{2+} signals (after inactivation of $I_{Ca,L}$): $\tau = 146$ ms before (a), 240 ms after flash (b). (B) Statistical analysis (from left to right): τ of Ca^{2+}-transient decay (2.26±0.51 of control),Ca^{2+}-transient amplitude (0.95±0.02 of control), peak $I_{Ca,L}$ (0.97±0.03 of control) and τ of $I_{Ca,L}$ inactivation (0.96±0.04 of control). (*: $p<0.05$, $n=8$ cells).

pH 7.2 (adjusted with CsOH). External solution (mM): NaCl 140, KCl 5, glucose 10, $CaCl_2$ 1.8 (or 6), CsCl 1, $BaCl_2$ 0.5, HEPES 10, pH 7.4 (adjusted with NaOH). Puffs of this solution containing 10 mM caffeine were applied to estimate SR Ca^{2+} content where indicated. Inhibition of the SERCA by photoreleased DBHQ was evaluated by analyzing the decay of the $[Ca^{2+}]_i$-transient during long lasting (1500 ms) depolarizations (to minimize Ca^{2+}-efflux *via* Na^+/Ca^{2+} exchanger). Membrane currents were recorded with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA), data were acquired using custom written software developed under LabView (National Instruments, Austin,

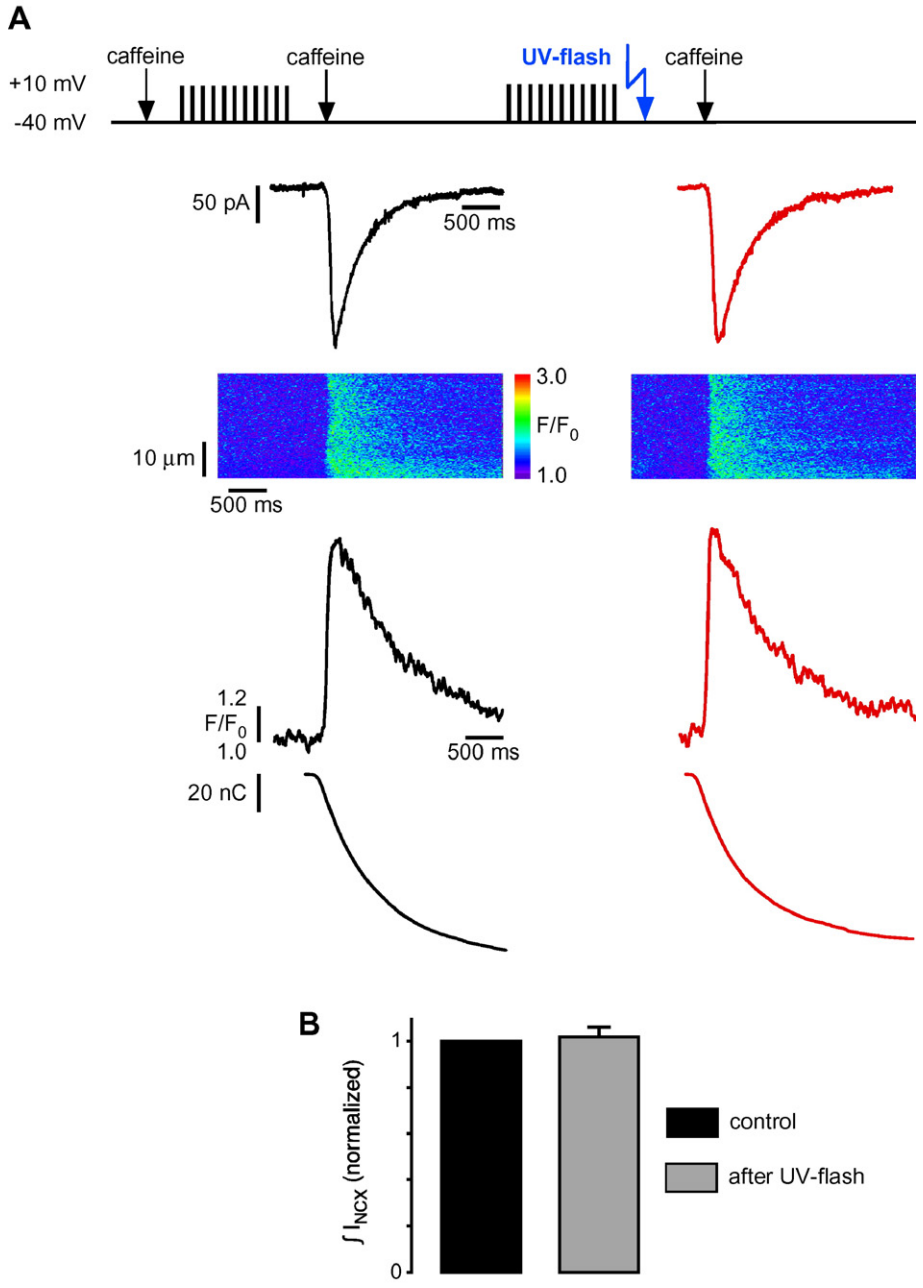

Fig. 3. The SR Ca^{2+} content is not reduced within seconds after photorelease of DBHQ. (A) Example recording of Na–Ca exchange current (I_{NCX}) during rapid caffeine application (10 mM for 2 s) before (left) and after DBHQ photorelease (right) in 1.8 mM extracellular [Ca^{2+}]. From top to bottom: experimental protocol, I_{NCX}, line-scan image, fluorescence changes, integrated I_{NCX}, representing amount of Ca^{2+} released from the SR. (B) Statistical analysis of normalized SR Ca^{2+} content after UV-flash was 1.04±0.02 of control (n=4 cells).

TX). Data analysis was carried out with IgorPro software (WaveMetrics, Lake Oswego, OR).

2.3. Confocal calcium imaging

Fluo-3 was excited at 488 nm line of an argon laser (50 µW) and fluorescence (>515 nm) was collected with a PMT on an MRC-100 confocal microscope (Bio-Rad). Image analysis was carried out with NIH-Image-SXM. Fluorescence signals are expressed as normalized fluorescence F/F_0. For details of the image acquisition and flash photolysis see [12]. Amplitude information was extracted from line-scan images after aligning the scanned lines to correct for the signal distortion due to the slow wave propagation. Statistical analysis of the Ca^{2+} signals: *: $p<0.05$; **: $p<0.01$, one-way ANOVA followed by Dunnett's test, mean±SE.

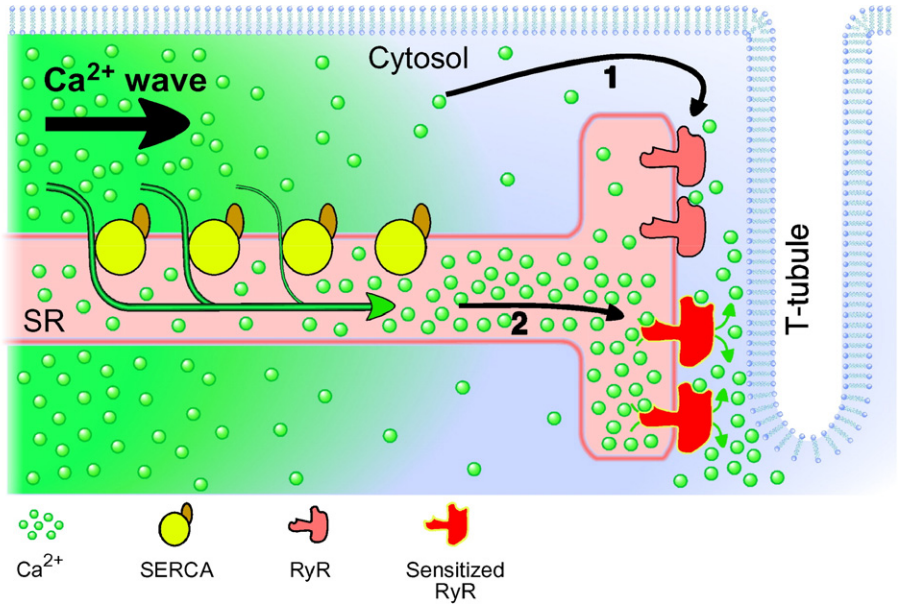

Fig. 4. Possible mechanisms for Ca^{2+} wave propagation acting in tandem. 1: Ca^{2+} waves driven by CICR; the elevated $[Ca^{2+}]_i$ in the wave-front induces Ca^{2+} release from the SR via RyRs. 2: Wave of RyR sensitization mediated by Ca^{2+} pumping into the SR by the SERCA. Diffusion of free Ca^{2+} inside the longitudinal SR results in slight elevations of $[Ca^{2+}]_{SR}$ ahead of the cytosolic wave, leading to RyR sensitization for cytosolic Ca^{2+} triggers. Rapid Ca^{2+} diffusion inside the SR and the resulting sensitization of the RyRs are driving the wave.

2.4. UV-flash photolysis

For photolysis of Nmoc-DBHQ UV-flashes from a Xenon short-arc flashlamp (wavelength 340–390 nm, flash duration 400 μs) were coupled into the microscope via an optical light-guide [12]. Spontaneous Ca^{2+} waves were induced by increasing the extracellular Ca^{2+} concentration to 6 mM.

3. Results

To assess whether Ca^{2+} waves in cardiomyocytes are predominantly driven by CICR directly or by a sensitization wave inside the SR, we used UV-flash photolytic liberation of the SERCA inhibitor DBHQ from a "caged" precursor, Nmoc-DBHQ [13]. Spontaneous Ca^{2+} waves were evoked by elevating the extracellular Ca^{2+} concentration to 6 mM. Photolysis of Nmoc-DBHQ during Ca^{2+} waves rapidly (i.e. within 1 s) slowed the propagation from around 95 μm/s to 69 μm/s, as revealed by laser-scanning confocal microscopic Ca^{2+} imaging (Fig. 1A). To determine the extent of SERCA block that can be achieved with one photolytic flash we assessed the slowing of Ca^{2+} transient decays in 100 μM of the SERCA inhibitor TBQ applied extracellularly, a concentration which blocks the Ca^{2+} pump completely. By comparison with the slowing observed after a single UV-flash we estimated the degree of SERCA inhibition by photoreleased DBHQ to be around 31%. Such an immediate deceleration of Ca^{2+} wave-propagation is consistent with Ca^{2+} uptake via the SERCA mediating a wave-front of RyR sensitization traveling inside the SR. Unbound luminal Ca^{2+} diffuses rapidly inside the longitudinal SR to the dyads of the next sarcomere, as it has been reported very recently [14]. This leads to RyR sensitization ahead of the cytosolic Ca^{2+} wave, which travels more slowly and mainly by diffusion of Ca^{2+} bound to mobile Ca^{2+} buffers [15]. A consequence of this sensitization is that a less pronounced elevation of $[Ca^{2+}]_i$ is required at the next sarcomere, such that RyRs will open earlier and even with the moderate $[Ca^{2+}]_i$ present at the Ca^{2+} wave-front [2,10]. The opposite experimental result – an acceleration of the propagation – would be expected after the UV-flash for Ca^{2+} waves mainly driven by CICR since, after SERCA inhibition, less Ca^{2+} is removed from the wave-front and more remains in the cytosol to trigger CICR. An alternative mechanism could result from sensitization of RyRs after SERCA mediated SR Ca^{2+} uptake taking place in the same site where the release occurs, since SERCAs are also present in the junctional SR. This mechanism would not require fast diffusion of SR Ca^{2+}. But it seems somewhat less likely because the SR Ca^{2+} pumps have low turnover rates (5–7 s^{-1} [16]), which would not allow for sufficient time to sensitize the RyRs before they are exposed to cytosolic trigger Ca^{2+}.

Previous studies of Ca^{2+} wave propagation using conventional pharmacological inhibition of the SERCA have provided contradictory results. Inhibiting the SERCA with the irreversible blocker thapsigargin led to an acceleration of the waves [17], while application of the SERCA blocker DBHQ (a.k.a. TBQ) resulted in a slowing of wave propagation [18]. While the exact reason for this discrepancy is still elusive, several confounding factors need to be considered. The prolonged SERCA inhibition, used in these pharmacological experiments, is known to cause secondary, long-term changes in cell physiology, including elevations of $[Ca^{2+}]_i$, loss of SR Ca^{2+} content,

impaired SR Ca^{2+} release and changes of the L-type Ca^{2+} current. However, with the rapid flash photolytic liberation of a SERCA inhibitor used in the present study, these secondary changes are expected to be minimal in the short time-frame of our experiments. Indeed, none of the parameters known to change during prolonged experiments were significantly affected in our study using a flash photolytic approach, except when applying series of flashes over more prolonged times. As expected, however, the time-course of the Ca^{2+} decay trailing the wave was immediately slowed. To exclude changes of the SR Ca^{2+} release mechanism and the Ca^{2+} current by photo-released DBHQ we recorded Ca^{2+} transients triggered by L-type Ca^{2+} currents (Fig. 2). Both parameters remained unchanged, only the late phase of the Ca^{2+} transient decay during the voltage-clamp depolarization was significantly slowed. While the early phase of the Ca^{2+} transient contains contributions from the inactivating Ca^{2+} current, the late phase is governed by SR Ca^{2+} uptake via the SERCA, and therefore this immediate slowing was expected. Also the SR Ca^{2+} content did not change noticeably, as confirmed by recording Ca^{2+} transients and Na–Ca exchange currents induced by puffs of caffeine before and after the flash (Fig. 3). Taken together, the observed changes of the Ca^{2+} decline were precisely what we had anticipated, since they reflect immediate consequences of SERCA inhibition. Furthermore, these experiments provide evidence that neither DBHQ nor other photolytic by-products of the caged compound interfered with L-type Ca^{2+} current, CICR, or SR Ca^{2+} content.

4. Discussion

The observation that an SR luminal mechanism can sustain Ca^{2+} wave propagation is not entirely unexpected. First of all, it may be anticipated based on several recent reports linking CICR termination to functional SR Ca^{2+} depletion [12,19,20]. In cardiac muscle, CICR must terminate after each heartbeat to ensure muscle relaxation. Among several proposed mechanisms for CICR termination, functional SR Ca^{2+} depletion has recently gained strong experimental support. It appears that Ca^{2+} unbinding from the intra-SR Ca^{2+} buffer calsequestrin makes the RyRs less sensitive to *cytosolic* Ca^{2+} [21], via a retrograde signaling pathway from calsequestrin to the RyRs, presumably mediated by the small accessory SR proteins triadin and junctin [22]. Thus, overloading the SR with Ca^{2+} would be expected to lead to a "sensitization" of the RyRs. In fact, it is well established that elevations of SR Ca^{2+} content increase fractional SR Ca^{2+} release in a way that is steeply Ca^{2+}-dependent [23]. This implies that even a small increase in $[Ca^{2+}]_{SR}$ in the region of the Ca^{2+} wave-front would be sufficient to sensitize the RyRs locally, while on the level of the entire cell this would not change the $[Ca^{2+}]_{SR}$ noticeably. In contrast, in myocytes with a normal SR Ca^{2+} load the local Ca^{2+} signals are known not to propagate as waves, indicating that Ca^{2+} overload is required [24].

Ca^{2+} waves are known to occur in many cell types. Interestingly, when comparing different varieties of Ca^{2+} waves in many systems and over a broad range of conditions, the waves in cardiomyocytes have consistently been found to exhibit extraordinarily fast propagation [6]. Based on this observation it had been suspected that a luminal propagation mechanism may contribute to wave propagation, acting in tandem with CICR (see Fig. 4).

Taken together, the findings reported here are contrary to predictions derived from the classical view of Ca^{2+} wave propagation by CICR, but entirely consistent with the concept that SR Ca^{2+} loading *via* SERCA leads to sensitization of the RyRs, thereby creating a traveling wave of high Ca^{2+} sensitivity initiating Ca^{2+}-release. Thus, in cardiac muscle, and presumably in many other cell types, Ca^{2+} wave propagation appears to be driven by a traveling wave-front of CICR "sensitization" rather than by CICR alone. This fundamental mechanism of RyR activation is most likely also crucial for our understanding of life threatening cardiac arrhythmias triggered by spontaneous Ca^{2+} releases, since it can render the RyRs abnormally sensitive for $[Ca^{2+}]_i$ and thus lowers the threshold for regenerative Ca^{2+} release events [25]. This type of arrhythmia is linked to RyR hyperphosphorylation [26], or to the recently discovered mutations of the RyR [1], or of the Ca^{2+} buffering protein inside the SR (calsequestrin [27]).

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