Role of Sarcomere Mechanics and Ca^{2+} Overload in Ca^{2+} Waves and Arrhythmias in Rat Cardiac Muscle

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ABSTRACT: Ca^{2+} release from the sarcoplasmic reticulum (SR) depends on the sarcoplasmic reticulum (SR) Ca^{2+} load and the cytosolic Ca^{2+} level. Arrhythmogenic Ca^{2+} waves underlying triggered propagated contractions arise from Ca^{2+} overloaded regions near damaged areas in the cardiac muscle. Ca^{2+} waves can also be induced in undamaged muscle, in regions with nonuniform excitation–contraction (EC) coupling by the cycle of stretch and release in the border zone between the damaged and intact regions. We hypothesize that rapid shortening of sarcomeres in the border zone during relaxation causes Ca^{2+} release from troponin C (TnC) on thin filaments and initiates Ca^{2+} waves. Elimination of this shortening will inhibit the initiation of Ca^{2+} waves, while SR Ca^{2+} overload will enhance the waves. Force, sarcomere length (SL), and $[Ca^{2+}]_i$ were measured and muscle length was controlled. A small jet of Hepes solution with an extracellular $[Ca^{2+}]$ 10 mM (HC), or HC containing BDM, was used to weaken a 300 μm long muscle segment. Trains of electrical stimuli were used to induce Ca^{2+} waves. The effects of small exponential stretches on triggered propagatory contraction (TPC) amplitude and propagation velocity of Ca^{2+} waves (V_{prop}) were studied. Sarcomere shortening was uniform prior to activation. HC induced spontaneous diastolic sarcomere contractions in the jet region and attenuated twitch sarcomere shortening; HC+ butanedione monoxime (BDM) caused stretch only in the jet region. Stimulus trains induced Ca^{2+} waves, which started inside the HC jet region during twitch relaxation. Ca^{2+} waves started in the border zone of the BDM jet. The initial local $[Ca^{2+}]_i$ rise of the waves by HC was twice that by BDM. The waves propagated at a V_{prop} of 2.0 ± 0.2 mm/sec. Arrhythmias occurred frequently in trabeculae following exposure to the HC jet. Stretch early during relaxation, which reduced sarcomere shortening in the weakened regions, substantially decreased

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force of the TPC (F_{TPC}) and delayed Ca^{2+} waves, and reduced V_{prop} commensurate with the reduction F_{TPC}. These results are consistent with the hypothesis that Ca^{2+} release from the myofilaments initiates arrhythmogenic propagating Ca^{2+} release. Prevention of sarcomere shortening, by itself, did not inhibit Ca^{2+} wave generation. SR Ca^{2+} overload potentiated initiation and propagation of Ca^{2+} waves.

KEYWORDS: **arrhythmogenic; Ca^{2+} waves; shortening; SR; Ca^{2+} overload.**

INTRODUCTION

Currently proposed molecular mechanisms underlying sarcomere length (SL) dependence of Ca^{2+} binding to troponin C (TnC) suggest that cross-bridge force exerted on the actin filament deforms the TnC molecule, thus retarding the dissociation of Ca^{2+} from TnC.[1,2] This effect is bound to be SL-dependent since the number of myosin cross-bridges attaching to actin increases with SL over the range of operation of cardiac muscle. Thus, the mechanical load on a sarcomere will influence the dissociation of Ca^{2+} from TnC. In fact, it has been shown that rapid removal of an external load on a muscle during the twitch causes a robust additional $[Ca^{2+}]_i$ transient.[3] This phenomenon may be important when the ECC properties of the myocardium are nonuniform (such as in disease), since nonuniformity of contraction may be accompanied by such unloading-related $[Ca^{2+}]_i$ transients. It is well known that a large amount of Ca^{2+} is bound to TnC during contraction. Hence, Ca^{2+} release from TnC upon rapid unloading may be large enough to activate Ca^{2+}-dependent mechanisms including SR Ca^{2+} release and Ca^{2+}-dependent membrane currents.

REVERSE EXCITATION–CONTRACTION (EC) COUPLING IN A NONUNIFORM CARDIAC MUSCLE

During our work on cardiac sarcomere dynamics we have discovered that when cardiac muscle is damaged locally, Ca^{2+} waves start near the damaged region and propagate rapidly in a coordinated fashion into adjacent tissue.[4] These aftercontractions in multicellular preparations occur as the combined result of the mechanical effects and elevated cellular $[Ca^{2+}]_i$ levels owing to the regional damage and thus may give rise to premature beats as well as triggered arrhythmias. These aftercontractions appear to be initiated by stretch and release of the damaged region during the regular twitch and they propagate into neighboring myocardium; hence the term triggered propagated contractions (TPCs). Damage-induced TPCs may, therefore, serve as the mechanism that couples regional damage with the initiation of premature beats and arrhythmias in the adjacent myocardium. The displacement of the TPC occurs at a velocity of propagation (V_{prop}), which varies at room temperature from

0.1 to 15 mm/sec[5] and is correlated tightly with the amplitude of the twitch preceding the TPC, suggesting that the Ca^{2+} load of the sarcoplasmic reticulum (SR) dictates V_{prop}. In contrast, sarcomere stretch, which increases the twitch force for any level of loading of the SR, does not increase V_{prop} of the TPC.[6] Studies of the effects of interventions such as varied $[Ca^{2+}]_o$, Ca^{2+} channel agonists and antagonists also support the idea that the SR Ca^{2+} load is an important determinant of V_{prop}.[7] On the other hand, interventions that cause a leak of Ca^{2+} from the SR (caffeine and ryanodine) increase V_{prop}, suggesting that V_{prop} also depends on diastolic cytosolic $[Ca^{2+}]_i$.[8] Finally, the rate of initiation of TPCs is tightly correlated with V_{prop} when the SR Ca^{2+} load is modulated, suggesting that the triggering process and the propagation process share closely related mechanisms.

We have recently investigated Ca^{2+} waves underlying TPCs in rat's cardiac trabeculae under experimental conditions that simulate the functional nonuniformity caused by local mechanical or ischemic damage of the myocardium. A mechanical discontinuity along the trabeculae was created by exposing the preparation to a small jet of solution with a composition that reduces ECC in myocytes within that segment. The jet solution contained either caffeine (CF), 2,3-butanedione monoxime (BDM), or low Ca^{2+} concentration ($[Ca^{2+}]$) (LC). Each of these solutions was chosen to render the exposed segment weaker than the normal muscle parts, by either depleting the SR of Ca^{2+} ions (CF) or inhibiting the cross-bridges (BDM) or by lowering the Ca^{2+} load of the cell (LC). The jet of solution was applied perpendicularly to a small muscle region (200 to 300 μm) at constant flow. When the jet contained caffeine, BDM, or low $[Ca^{2+}]$ during the stimulated twitch, muscle-twitch force decreased and the sarcomeres in the exposed segment were stretched by shortening normal regions outside the jet. Repeated stimulation at 2.5 Hz, room temperature, and physiological $[Ca^{2+}]_o$, reproducibly generated Ca^{2+} waves that arose from the border between shortening and stretched regions. Such Ca^{2+} waves started during force relaxation of the last stimulated twitch of the train and propagated into segments both inside and outside of the jet. Arrhythmias, in the form of nondriven rhythmic activity, were induced when the amplitude of the Ca^{2+} -wave was increased by raising $[Ca^{2+}]_o$. Arrhythmias disappeared rapidly when the uniformity of ECC throughout the muscle was restored by turning the jet off. These results showed, for the first time, that nonuniform ECC can cause Ca^{2+} waves underlying TPCs and suggest that Ca^{2+} dissociated from myofilaments plays an important role in the initiation of Ca^{2+} waves.

SPONTANEOUS SR CA^{2+} RELEASE

Normal Ventricular Muscle

Spontaneous release of Ca^{2+} from the SR is evident in the form of Ca^{2+} "sparks,"[9] which are, as "evoked Ca^{2+} sparks" induced during action

potentials,[10] or voltage-clamp pulses,[11] probably by Ca^{2+} entering via single L-type Ca^{2+} channels.[12–14] Ca^{2+} sparks may also trigger each other to produce Ca^{2+} waves, which propagate through the cell.[15] Ca^{2+} sparks evoked by L-type Ca^{2+} currents are believed to summate, spatially and temporally, constituting the electrically evoked whole cell $[Ca^{2+}]_i$ transient[10–13,16] that couples excitation to contraction. The relevance of Ca^{2+} sparks to normal ECC in cardiac muscle was proven by similar observations using confocal microscopy of working ventricular trabeculae under physiological conditions ($[Ca^{2+}]_i$ and temperature). Also, microscopic Ca^{2+} waves were found in these trabeculae, which had been recorded previously only in single isolated cells.[17–19] The peak amplitude Ca^{2+} sparks is ~200 nmol/L, which is below the level at which cross-bridges are activated in the intact trabeculae. Furthermore, Ca^{2+} sparks are spatially restricted, suggesting that the $[Ca^{2+}]$ in the myofilament space during and after the peak of the Ca^{2+} spark must have been substantially lower than 170 nM, which makes it even more unlikely that cross-bridges are activated by individual Ca^{2+} sparks. The observation that Ca^{2+} sparks and micro-Ca^{2+} waves occur in microscopically quiescent muscles is therefore not surprising.

SR Ca^{2+} Overload and Spontaneous Ca^{2+} Release

Spontaneous SR Ca^{2+} release was first observed by Fabiato and Fabiato[20] in the form of spontaneous oscillatory contractions in skinned myocyte fragments. The spontaneous contractions were initiated by loading the SR with Ca^{2+}, while the $[Ca^{2+}]$ used for the loading was by itself insufficient to induce Ca^{2+} release. This observation led to the concept that a heavily Ca^{2+}-loaded SR is characterized by spontaneous Ca^{2+} release.[21] The mechanism for increased probability of opening of the SR- Ca^{2+}channel when the SR is heavily loaded with Ca^{2+} is still uncertain, but suggests that the channel is directly or indirectly sensitive to the luminal $[Ca^{2+}]$ of the SR. Intact cells with a high SR Ca^{2+} load show similar phenomena.[22,23]

Spontaneous SR Ca^{2+} release in intact multicellular preparations can take on forms that range from an increased rate of Ca^{2+} spark generation to Ca^{2+} waves that propagate throughout individual cells accompanied by propagating contractile waves[24] or even waves that traverse borders between cells and ultimately repetitive oscillatory Ca^{2+} release occurring synchronously throughout the preparation. The importance of this phenomenon is that the propensity of cardiac muscle in CHF, as was shown in rat, may generate spontaneous cellular contractile waves at increased stimulation frequency or following catecholamine activation. This phenomenon appeared to result in a decrease in force of the driven contraction. These spontaneous contractile waves render failing cardiac muscle unable to augment force in response to increased heart rate and sympathetic stimulation.[24]

Another important observation has been that spontaneous cytosolic $[Ca^{2+}]_i$ transients[25,26] are accompanied by spontaneous depolarizing transmembrane currents in single myocytes as well as in nondriven multicellular cardiac preparations.[23,27] The resulting depolarization may be large enough to trigger an action potential. Ca^{2+} entry during the ensuing action potential may cause even more Ca^{2+} loading of the SR. Consequently, as soon as the release process has recovered after the electrically induced Ca^{2+} release, the overloaded SR again releases a fraction of its Ca^{2+} leading to another action potential and a so-called triggered arrhythmia. Agents that reduce Ca^{2+} load of the SR (e.g., ryanodine, caffeine, EGTA buffer) abolish spontaneous $[Ca^{2+}]_i$ oscillations as well as the oscillatory depolarizations and contractions.[28,29] Therefore, it is thought that spontaneous $[Ca^{2+}]_i$ oscillations are not secondary to transmembrane potential changes, but may cause depolarization and give rise to nondriven action potentials.[30,31]

The Aim of this Study

It is clear that mechanical nonuniformity of cardiac muscle can be induced by mechanisms (e.g., local differences in catecholamine levels or local ischemia) that differ from nonuniformity due to spontaneous Ca^{2+} release (e.g., local postischemic damage). What we do not know is the contribution and/or interaction of these two mechanisms of nonuniformity to the induction of arrhythmogenic Ca^{2+} waves.

The purpose of the studies reported here was to explore whether local Ca^{2+} overload induces Ca^{2+} waves in nonuniform myocardium and whether the reduction of force generating capacity of Ca^{2+} overloaded cardiac muscle during the electrically driven twitch is involved in the generation of propagated Ca^{2+} waves in normal myocardium.

METHODS

Dissection of the Trabeculae

For the studies of the effect of reduced contractile force in the presence of an overloaded SR, 14 trabeculae were dissected from the right ventricle of Lewis Brown Norway rats (length 2.5 ± 0.13, width 0.25 ± 0.03, thickness 0.10 ± 0.01 mM). For the studies of the effect of muscle length, 17 muscles (length: 2.26 ± 0.11 mM, width: 227 ± 18 μM, thickness: 114 ± 7 μM in slack conditions) were used.[32,33] The muscles were mounted in a bathe on an inverted microscope (Nikon Canada Inc., Missisauga, Ontario, Canada) between a silicon strain gauge and a servo-controlled motor arm. Force and

SL were measured respectively by the strain gauge and laser diffraction techniques.[34] Muscle length was changed using a servo-controlled motor and set at 2.0 μM of resting SL (SL_0). The muscle was stimulated in HEPES buffer solution ((in mM): 137.2 NaCl, 5KCl, 1.2$MgCl_2$ 2.8 Na-Acetate, 1 Taurine, 10 HEPES, 10 Glucose, $[Ca^{2+}]_o = 0.7$, 100% O_2 at pH 7.4. at 25°C) at 0.5 Hz until contractions were stable.

Local Jet Exposure

To suppress regional activation of the muscle, the restricted region was exposed to a small "jet" of solution as reported previously.[33] Briefly, the jet was continuously applied using a syringe pump (≈ 0.06 mL/min) from a glass pipette (≈ 100 μm tip) perpendicularly to the trabecula (FIG. 1 A). Alignment and position of the jet flow with the muscle was adjusted using fluorescein (< 0.01 mg/mL). The jet solution was composed of standard HEPES solution containing: (1) caffeine (5 mmol/L) to deplete Ca^{2+} content in the SR[35,36] and (2) 2,3-butanedione monoxime (BDM; 20 mmol/L) to suppress the activation of cross-bridges.[37–40] Ca^{2+} concentrations in the jet ($[Ca^{2+}]_{jet}$) were set at 2.0 mmol/L, as those in the bath ($[Ca^{2+}]_o$). Using this method, sarcomeres in the jet-exposed region were stretched during the stimulated twitches (FIG. 1 B) as has been shown previously.[33]

The effect of the combination of Ca^{2+} overload of the SR was studied by exposing the muscle to a jet of a standard HEPES solution with added Ca^{2+}: high (10 mM) $[Ca^{2+}]_o$ (HC jet) or the combination of HC and 2,3-butanedione monoxime (BDM;20 mM) to inhibit cross-bridge action (HC+BDM jet).

Fura-2 Loading and $[Ca^{2+}]_i$ Measurement

Measurements of cytosolic Ca^{2+} concentration in trabeculae ($[Ca^{2+}]_i$) have been described in detail.[32,41] Briefly, Fura-2 salt was microinjected iontophoretically into the trabecula. Fluorescence signals evoked by excitation lights of 340, 360, or 380 nm were collected using (1) a photomultiplier tube (PMT), which provides average regional $[Ca^{2+}]_i$ using 340/380 nm ratio and *in vitro* calibration or (2) an image intensified CCD camera (IIC) at 30 frames/sec to assess local $[Ca^{2+}]_i$.

The local $[Ca^{2+}]_i$ was analyzed from fluorescence images (8 bit BMP, 512 × 512 pixels; pixel size:2.94 × 2.17 μM), which consisted of one fluorescence image at 360 nm in the resting condition (Im_{Ref}) and a sequence of images at 380 nm.[42] (Im_{380}) recorded continuously during stimulus protocols. The overall kinetics of $[Ca^{2+}]_i$ were obtained from pixel-to-pixel ratios Im_{Ref}/Im_{380} (ratio images) after subtraction of the autofluorescence. Longitudinal ratio profiles

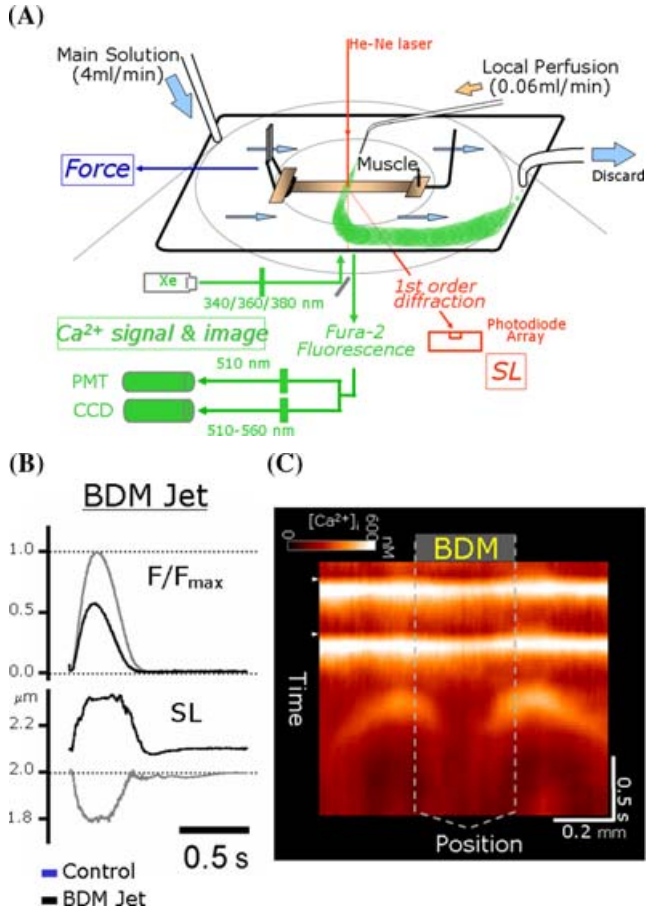

FIGURE 1. Panel **A** shows the experimental paradigm to study nonuniform contraction in rat cardiac trabeculae. The muscle is mounted in an epifluorescent microscope and exposed to a HEPES solution flowing through the bath, together with a jet of modified HEPES solution (see text) that renders the segment in the jet weaker than the remainder of the muscle. SL is measured by laser diffraction, F by strain gauge, and $[Ca^{2+}]_i$ by Fura fluorescence using a PMT and image-intensified camera (CCD). Panel **B** shows the effects of a local jet with BDM: a jet containing BDM inhibits the activation of sarcomeres in the jet resulting in regional stretch of the muscle in the jet. Panel **C**. The spatiotemporal Ca^{2+} distribution shows that Ca^{2+} waves arise from the border between regions with and without the BDM jet following a repeated stimulation (2.5 Hz–7.5 sec). Arrowhead: moment of electrical stimulation.

along the long axis of the trabecula were calculated from the mean ratio across the region of interest (ROI: 250–300 × 25 pixels) and then expressed as $[Ca^{2+}]_i$ using linear regression between PMT and IIC ratio.[43] Finally, variations in local $[Ca^{2+}]_i$ along the trabeculae were studied through spatiotemporal diagrams as represented in FIGURE 1 C.

Protocols

We have studied the effect of various interventions on Ca^{2+} waves, which were induced by stimulus trains (2.5 Hz for 7.5 sec, repeated every 15 sec; $[Ca^{2+}]_o = 2.0$ mmol/L; 23.5°C[43]). Ca^{2+} overload was induced using 0.5 Hz pacing at varied $[Ca^{2+}]_o$ of 0.2–10 mM and the effect of the HC jet by measurement of regional SL during 0.5Hz pacing (25°C, $[Ca^{2+}]_o = 0.7$ mM) and quantification of Ca^{2+} waves following the stimulus trains $[Ca^{2+}]_{BATH} =$ 2.0 mM.

The effects of SL on F and regional SL inside the jet containing caffeine or BDM were tested during 0.5 Hz regular stimulus at room temperature (24.4 ± 0.4°C). The muscle length was set at 1.9, 2.0, or 2.1 μM of SL_0 before the jet exposure. In 12 trabeculae, we studied the effects of length changes on initiation and propagation of Ca^{2+} waves induced by exposing of the muscle to the jet containing caffeine or BDM. During exposure to the jet solutions Ca^{2+} waves were induced by the rapid stimulus train at SL 2.0 μM. When stimulated twitches and Ca^{2+} waves became steady, the SL was changed from 2.0 to 2.1 μM or to 1.9 μM and the $[Ca^{2+}]_i$ was measured using IIC before and less than 1 min after the length changes.

Data Analysis

Data were expressed as mean ± SEM. Statistical analysis was performed using unpaired *t*-test or (analysis of variance) ANOVA followed by a *post hoc* test. Differences were considered as significant when $P < 0.05$.

RESULTS

Ca^{2+} Waves Initiated in a Muscle Region Exposed to High $[Ca^{2}]_0$

As we have shown before, sarcomeres shortened (~12%) uniformly in these trabeculae during superfusion with normal HEPES solution. Muscle-twitch force decreased and the sarcomeres in the exposed segment were stretched by shortening the normal regions outside the jet when the jet contained caffeine, BDM, or low-$[Ca^{2+}]$.[2,33] During relaxation, the sarcomeres in the exposed segment shortened rapidly. Short trains of stimulation at 2.5 Hz reproducibly caused Ca^{2+} waves to arise from the borders exposed to the jet. These Ca^{2+} waves started during force relaxation of the last stimulated twitch and propagated into segments both inside and outside of the jet. Arrhythmias, in the form of nondriven rhythmic activity, were triggered when the amplitude of the Ca^{2+} wave was increased by raising $[Ca^{2+}]_o$. The arrhythmias disappeared when the muscle uniformity was restored by turning the jet off.[33]

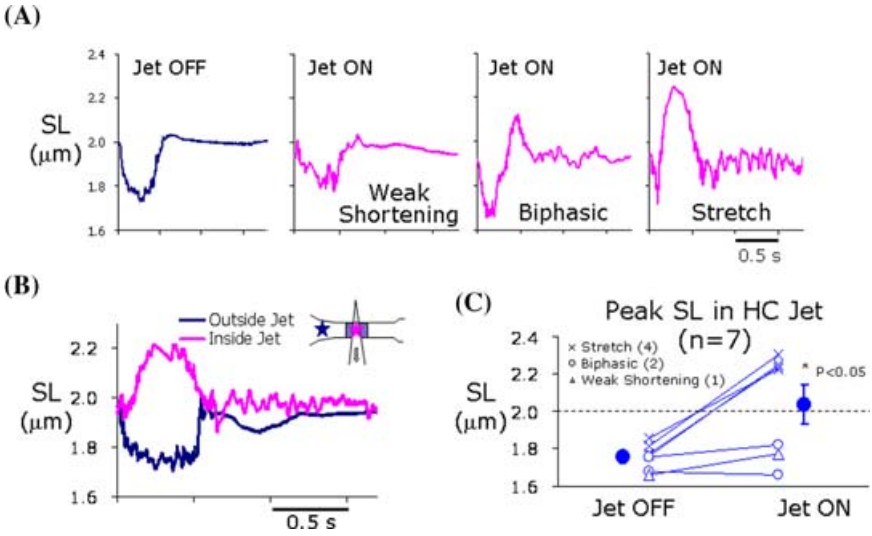

FIGURE 2. Panel **A.** Regional SL recordings in a jet with a high $[Ca^{2+}]_o$ (see text) show reduced shortening, biphasic, and stretch patterns with fluctuating resting SL during jet exposure (Jet ON). Panel **B.** Simultaneous SL recordings inside and outside the HC jet, showing nonuniform sarcomere shortening in the trabecula. Panel **C.** Summary of peak SL in the HC jet before (off) and during (on) the jet exposure. 0.5 Hz pacing, $[Ca^{2+}]_o$ = 0.7 mM.

Exposure of the trabeculae to high $[Ca^{2+}]_o$ quite clearly caused Ca^{2+} overload of the SR because spontaneous contractile waves inside myocytes of the muscle were generated in the diastolic intervals between twitches. This spontaneous activity increased with external $[Ca^{2+}]_o$ and reduced force of the twitch following electrical stimuli in proportion to the probability of occurrence of spontaneous contractile events[24] (data not shown).

Similarly, exposure of the muscle segment in the jet to a high $[Ca^{2+}]_o$ in the medium consistently reduced force of the electrically stimulated (see e.g., FIG. 4). Exposure of the muscle segment in the jet to a high $[Ca^{2+}]_o$ in the medium reduced sarcomere shortening or turned the monophasic shortening pattern during the twitch into a pattern in which initial shortening was followed by stretch during the twitch, or into frank stretch during the whole twitch (FIG. 2). This sarcomere stretch occurred despite the fact that the $[Ca^{2+}]_i$ transient during the electrically stimulated twitch was increased (by $\sim$ 25 %) (FIG. 3).

The high $[Ca^{2+}]_o$ jet caused local $[Ca^{2+}]_i$ transients that started 500–600 msec after onset of the electrically evoked $[Ca^{2+}]_i$ transient when the muscle was stimulated at a relatively low stimulus rate (FIG. 3; 1 Hz). This interval was comparable to the interval between the Ca^{2+} surge that we have observed in muscle exposed to CF, BDM, or low $[Ca^{2+}]_o$ jets. When we exposed the

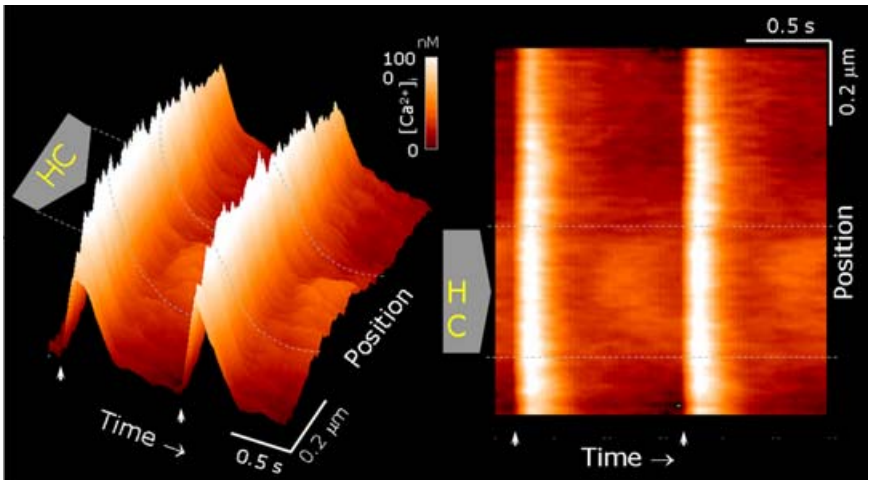

FIGURE 3. Ca^{2+} transients that are initiated in the region exposed to a high $[Ca^{2+}]$ jet. Three- (*left*) and corresponding two-dimensional (*right*) representations show regional $[Ca^{2+}]_i$ changes along the muscle during exposure to a high $[Ca^{2+}]$ jet (HC; *dotted lines*). Diastolic $[Ca^{2+}]_i$ rises in the jet region following Ca^{2+} transients are observed. Arrowhead indicates the moment of electrical stimulation (1Hz Pacing, $[Ca^{2+}]_o = 0.7$ mM calibrations as indicated).

muscles to stimulus trains, Ca^{2+} waves were generated; these Ca^{2+} waves now started in the center of the jet with high $[Ca^{2+}]_o$; this contrasts the behavior in muscle exposed to CF, BDM, or low $[Ca^{2+}]_o$ jets, where the Ca^{2+} waves invariably start in the border zone of the jet-exposed region.[33] Ca^{2+} waves starting in the high $[Ca^{2+}]_o$ jet region propagated with a similar V_{prop} (0.6–4.0 mm/sec) to the V_{prop} that we have described before for damaged muscle and for muscle in muscle exposed to CF, BDM, or low $[Ca^{2+}]_o$ jets.[33] It was striking in these experiments that the combination of a HC jet and repetitive stimulation with stimulus trains causes multiple nondriven $[Ca^{2+}]_i$ transients (FIG. 4).

It is likely that the oscillatory Ca^{2+} release that must have caused these transients may have been caused in part by Ca^{2+} overload of the SR.[44] It is not clear, therefore, what the contribution has been of a Ca^{2+} surge from the myofilaments compared to oscillatory Ca^{2+} release by the overloaded SR. We explored the contribution of oscillatory Ca^{2+} release by an overloaded SR compared to a Ca^{2+} surge from the myofilaments by suppressing cross-bridge activity using a jet solution in which the high $[Ca^{2+}]_o$ was combined with BDM. FIGURE 5 shows an example of the effects of this paradigm: oscillatory Ca^{2+} transients were still prominent, but the origin of the Ca^{2+} waves was now found (see start of the arrow in FIG. 5 B) in the border zone between the segment exposed to the jet and the segment of muscle remote from the jet. The wave initiation in HC+BDM occurred earlier than that in HC.

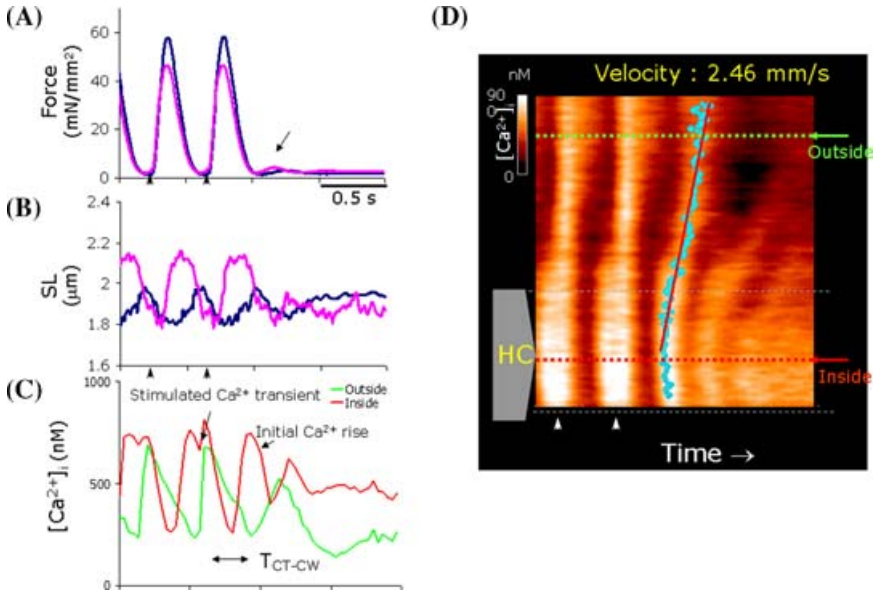

FIGURE 4. Ca^{2+} waves start in the region exposed to the HC jet. Panel **A.** Force (upper) and SL (lower) tracings showing that, during exposure of the HC jet, sarcomeres in the jet became stretched and a TPC (arrow) occurred, although the peak force of the twitches decreased. Panel **B.** Ca^{2+} waves were induced inside the HC jet region and propagated into the normal region. Panel **C.** Tracings of regional $[Ca^{2+}]_i$ inside and outside the HC jet region (shown in online version along red and green lines in panel C) during initiation of Ca^{2+} waves. Inside the HC jet, a large initial Ca^{2+} rise of the wave was observed (C_W = 743 nM), which reached 92% of the peak of the last Ca^{2+} transient (CT). Panel **D.** V_{prop} (2.46 mm/sec) was calculated by linear regression of displacement of the peaks of the Ca^{2+} wave. (2.5 Hz pacing, $[Ca^{2+}]_o = 2.0$ mM).

Ca^{2+} waves starting in the high $[Ca^{2+}]_o$ jet region tended to be faster than those in HC+BDM (1.2 ± 0.2; 0.5–1.8 mm/sec). The initial Ca^{2+} rise (CW) was large in both groups and corresponded to ≈90% of the peak stimulated Ca^{2+} transients (CT). A decrease of the initial Ca^{2+} rise (CW) may explain slower wave propagation in HC+BDM.

Arrhythmias could be induced in 4 of 10 muscles exposed to the jet of HC or HC + BDM by raising the frequency of the 7.5 sec stimulus train to 3 or 3.3 Hz-stimulus train. No arrhythmias could be induced in the absence of the jet.

Effects of Muscle Length on Force and Regional SL

If the requirement for the initiation of arrhythmogenic Ca^{2+} waves is that the region from where the Ca^{2+} waves start has a Ca^{2+}-loaded SR and exhibits active contraction owing to TnC activation of cross-bridges, albeit weaker than

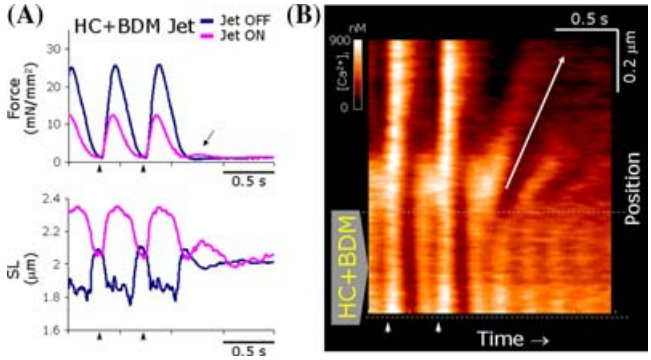

FIGURE 5. Ca^{2+} waves start in the border of the HC jet if contraction is eliminated by the addition of BDM to the jet solution. Panel **A.** Combination of HC and BDM in the jet reduced the peak force of twitches further and caused sarcomere stretch (lower) in the jet region. HC+BDM in the jet accelerated the TPC (arrow) and Ca^{2+} waves (Panel **B**), which now started in the region bordering the jet-exposed region where a large initial Ca^{2+} rise was observed. (2.5 Hz pacing, $[Ca^{2+}]_o = 2.0$ mM)

that of the neighboring muscle, one would expect that enhancing the amount of Ca^{2+} bound to TnC, for example, by stretch of the sarcomeres, would increase the Ca^{2+} surge during the relaxation phase and would accelerate Ca^{2+} waves. We tested this prediction in muscles exposed both to caffeine-containing jets and to BDM-containing jets.

Stretch (1.9–2.1 μM) increases force of the electrically stimulated twitches substantially without a noticeable change in the $[Ca^{2+}]_i$ transient.[45] The $[Ca^{2+}]_i$ transients in the jet as well as in the border zone and in the remote segments of the muscle were similar at 1.9, 2.0, and 2.1 μM (data not shown). Stretch increased the rate of decline of force during relaxation (FIG. 6) both in muscles that were uniformly superfused with HEPES solution and in muscles that were exposed to the caffeine and BDM jets.

The jet with caffeine or BDM reversibly decreased regional activation of sarcomeres in and around the jet, resulting in decrease in the peak force (∼50 % both using caffeine and BDM; FIG. 6 B) and sarcomere stretch in the jet-exposed region at each SL tested (1.9, 2.0, and 2.1 μM). During the jet exposure (with both BDM and caffeine), sarcomeres in the jet were stretched by stronger sarcomeres located outside the jet as reported previously.[2,33,46,47] As twitch force increased by lengthening of the muscle, the peak of sarcomere stretch in the jet-exposed region also increased up to ≈ 0.3 μM.

Effect of Length Changes on Ca^{2+} Waves

Ca^{2+} waves arose in the border zone at the edge of the segments exposed to the caffeine or BDM jets after the last-stimulated Ca^{2+} transient, and then

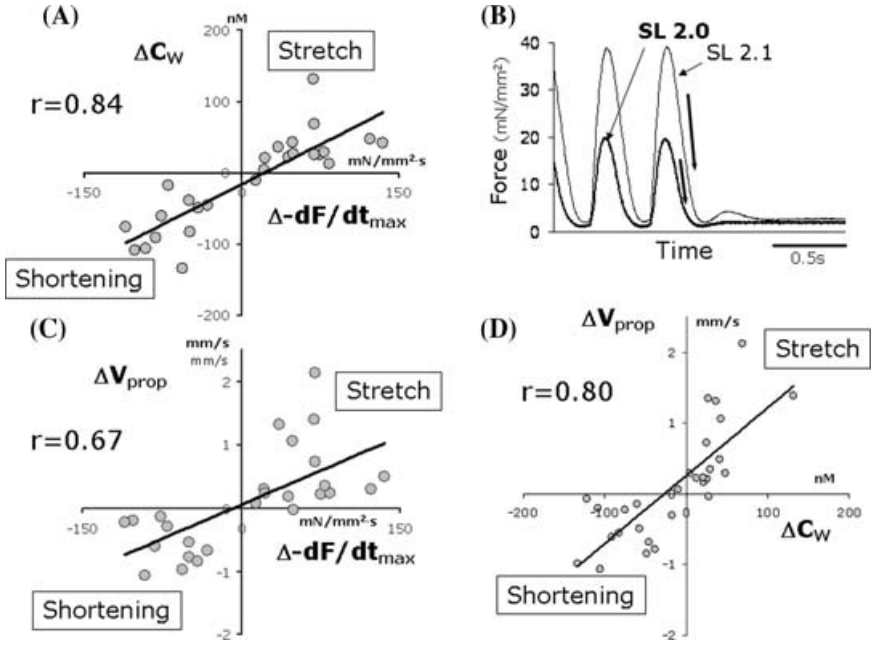

FIGURE 6. Accelerated force relaxation accelerates Ca^{2+}: Panel **B** shows that SL increase increases both twitch force and the rate of decline of twitch force. Panels **A** and **C** show that both amplitude of the initial $[Ca^{2+}]_i$ transient (ΔCw) in the border zone and V_{prop} of Ca^{2+} waves of BDM-exposed muscles are proportional to the rate of decline of the twitch at varied SL_o (range 1.9–2.1 μM; see text for details). Panel **D** shows that V_{prop} correlates tightly with ΔCw.

propagated into the normal muscle outside the jet as well as into the segment exposed to the BDM jet but not into the segment exposed to the caffeine jet.[33] Initiation of the Ca^{2+} waves occurred late during relaxation of the last stimulated twitch (see FIG. 6 A), corresponding to 26 $\pm$ 5% (caffeine; $n = 16$) and 23 $\pm$ 5% (BDM; $n = 11$) of peak force development at SL_0 2.0 μM, were similar to our previous data.[33] Lengthening the sarcomeres from 1.9 to 2.1 μM increased the propagation velocities (V_{prop}) of Ca^{2+} waves (0.98 to 2.00 mm/sec) in caffeine and from 1.14 to 2.66 mm/sec in BDM, together with an increase in the amplitude of the $[Ca^{2+}]_i$ transients during the waves.

We have studied the effect of length changes on initiation and propagation of Ca^{2+} waves separately. We calculated V_{prop} as well as the initial $[Ca^{2+}]_i$ rise (C_W) during initiation of Ca^{2+} waves. Muscle stretch (SL: 1.9–2.1 μm) significantly increased V_{prop} (0.54 $\pm$ 0.20 to 1.19 $\pm$ 0.17 mm/sec in caffeine and 0.53 $\pm$ 0.17 to 1.99 $\pm$ 0.62 mm/sec in BDM) and C_W (327 $\pm$ 25 to 351 $\pm$ 28 nmol/L in caffeine and from 312 $\pm$ 65 to 385 $\pm$ 38 nmol/L in BDM). SL did not affect SR Ca^{2+} loading (reflected by the peak of the last stimulated Ca^{2+} transient)[45] in the region of the wave initiation (i.e.,

border zone) and in the region where the Ca^{2+} wave propagated (i.e., outside the jet), suggesting that changes in C_W and V_{prop} were independent of those in regional Ca^{2+} loading. In contrast, V_{prop} correlated strongly and linearly with ΔC_W ($r = 0.8$, $P < 0.001$), independent of the composition of the jet solution, suggesting that changes in V_{prop} by the length changes depend on those in C_W, that is, the magnitude the initial $[Ca^{2+}]$ rise during the wave initiation.

Effect of Force Development and Relaxation on Ca^{2+} Waves

We have further explored the dependence of wave generation and propagation on the factors that dictate the initial rise of $[Ca^{2+}]_i$ at the initiation site. Our previous work has indicated that the initial $[Ca^{2+}]$ rise during the wave initiation is probably caused by Ca^{2+} dissociation from myofilaments as a result of quick release of active sarcomeres.[48] The amount of Ca^{2+} binding and dissociation from myofilaments by length changes has been shown to be correlated with those in the number of Ca^{2+}-activated cross-bridges; that is, force development of the myocardium.[49] Therefore, we compared the peak of force development (F_T) and the maximum rate of force relaxation ($-dF/dt_{max}$) during the last stimulated twitch with subsequent initiation and propagation of Ca^{2+} waves. F_T increased with stretch both and during exposure to jets with caffeine (4.6-fold) and BDM (3.1-fold) while $-dF/dt_{max}$ also showed similar changes (2.5- and 2.2-fold, respectively). Changes in ΔC_W and ΔV_{prop} of the Ca^{2+} waves correlated clearly with F_T induced by stretch as well as those in $-dF/dt_{max}$ (FIG. 6 A, C). These results strongly suggested that force development and relaxation of the twitch determine the initiation and subsequent propagation of Ca^{2+} waves.

Effect of Dynamic Stretch of the Muscle during Relaxation on Ca^{2+} Waves

The existence cooperativity of force and Ca^{2+} binding by TnC[1] predicts that the initial Ca^{2+} surge is proportional to the amount of Ca^{2+} bound to TnC and to the rate of force decline during relaxation. The rate of force decline is determined in the experiments by the interplay between the strong and weakened segments of the muscle. This interplay leads to rapid shortening of the weak but still contracting sarcomeres exposed to the jet. We tested whether the Ca^{2+} waves are modified by maintaining SL in the weakened segment at the length that was reached during the stretch during the twitch. Evidently, stretch early during the twitch enhances force owing to the positive slope of the F–SL relationship.

We have used exponential stretches (< 10% ML) to measure the amplitude of the TPC at the new SL; we corrected for length dependence force development of the TPC following the dynamic stretch by expressing the amplitude of the

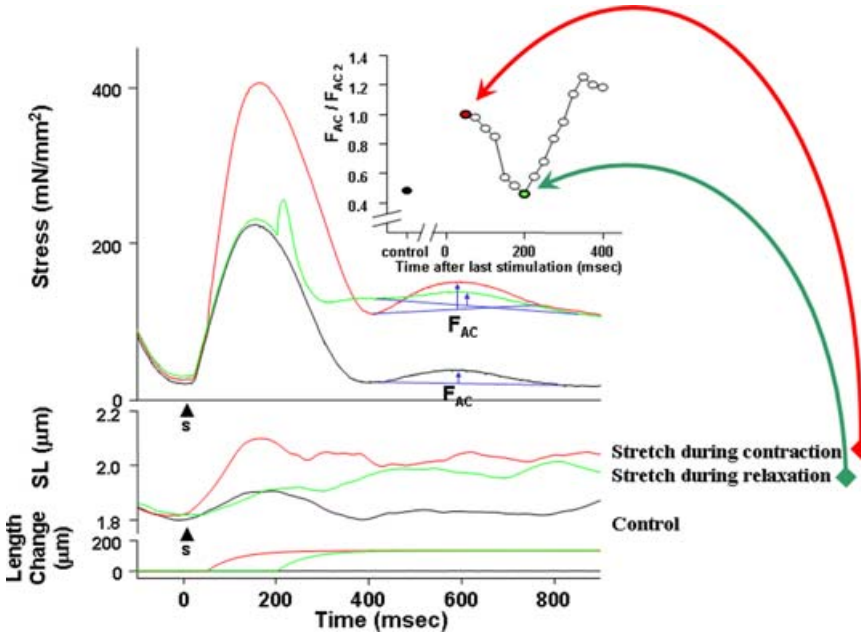

FIGURE 7. Elimination of sarcomere shortening in the border zone of the segment exposed to a BDM jet reduces but does not eliminate the TPC. The upper tracing shows three twitches: (1) the lower tracing at control SLo (1.8 μM; see bottom panel) when sarcomeres in the border zone are stretched by the stronger segments in the muscle [and shorten in the border zone during relaxation;] (2) the middle tracing in the top panel shows force when SL in the border zone is kept constant during relaxation by muscle stretch (starting at 200 msec; bottom panel); (3) a large twitch, which occurs when SL in the border zone is increased by a stretch at the beginning of the twitch. The force of the TPC (F_{AC}) of the control at short SL (1) is similar to the F_{AC} after stretch during relaxation (2) and reduced compared to (3) when the stretch was applied in the beginning of the twitch. The latter reduction (see inset) depended on the moment of the stretch and was proportional to the difference in twitch force between (2) and (3) (see inset).

force of the TPC as a fraction of the amplitude of the TPC following a twitch in which sarcomeres had been stretched at the onset of the twitch. FIGURE 7 top panel shows that the stretches indeed eliminated sarcomere shortening during relaxation. The stretches enhanced force transiently in proportion to the rate of SL increase. Still, maximal force during twitches remained similar to force at the short SL (1.8 μm) and never exceeded 60% of peak force at the greater SL. Accordingly, the amplitude of the TPC induced by elimination of shortening during the relaxation remained similar to TPC-amplitude at short SL 1.8 μM and was 50% of the amplitude of the TPC at SL 2.0 μM (FIG. 7 inset). The TPC following dynamic stretch during relaxation was smaller and started later than the TPC at the SL 2.0 μM following the early dynamic stretch.

DISCUSSION

These findings are consistent with previous observations in muscles exposed to a jet that renders the segment in the jet weaker than the neighboring muscle segments.[2,33] In those studies, we concluded that the requirements for the initiation of arrhythmogenic Ca^{2+} waves is that the region from where the Ca^{2+} waves start has a Ca^{2+}-loaded SR and exhibits active contraction, albeit weaker than that of the neighboring muscle. Exposure of the muscle to a high $[Ca^{2+}]_o$ reduces contractile force in proportion to an increase of spontaneous Ca^{2+} release by the overloaded SR, as has been shown both in normal cardiac muscle[50] and in muscle from failing rat heart.[51] Consistent with these previous studies we observed here that in muscle exposed to a HC jet indeed spontaneous diastolic Ca^{2+} release occurred and sarcomere shortening turned locally into stretch when the muscle was exposed to a jet of HC solution. Similar to the findings in muscles locally exposed to a jet that weakens contraction, it appeared that the cycle of stretch followed by shortening of the sarcomeres during relaxation of the normal segments of the muscle Ca^{2+} waves were initiated in the stretched region. Different from the findings in muscles locally exposed to a jet that weakens contraction, it was clear now that the Ca^{2+} waves started in the segment exposed to the HC jet and not in the border of the jet-exposed region. It is possible that the segment exposed to the HC jet generated such large SR Ca^{2+} overload-induced Ca^{2+} release that this spontaneous diastolic Ca^{2+} release alone was sufficiently large to account for the initiation of Ca^{2+} waves. This is unlikely, however, because inhibition of cross-bridge activity by HC+BDM in the jet shifted the site of origin of the Ca^{2+} waves to the border zone of the jet. Hence, the experiments using HC and HC+ BDM jets are consistent with the hypothesis that initiation of arrhythmogenic Ca^{2+} waves requires that the region from where the Ca^{2+} waves start has a Ca^{2+}-loaded SR and exhibits active contraction, albeit weaker than that of the neighboring muscle. Our experiments also suggest that SR Ca^{2+} overload facilitates the generation of Ca^{2+} waves.

We have previously proposed that the molecular mechanism underlying SL dependence of Ca^{2+} binding to TnC is that cross-bridge force exerted on the actin filament deforms the TnC molecule thus retarding the dissociation of Ca^{2+} from TnC.[2] This effect is bound to be SL-dependent since the number of myosin cross-bridges attaching to actin increases with SL over the range of operation of cardiac muscle. This hypothesis predicts that removal of an external load on a muscle during the twitch will cause a robust additional $[Ca^{2+}]_i$ transient as has been shown experimentally.[52] It is well known that a large amount of Ca^{2+} is bound to TnC during contraction and that the amount of Ca^{2+} bound to the TnC increases with sarcomere stretch owing to the increased number of cross-bridges that interact with actin in the stretched sarcomere. The increased binding of Ca^{2+} to TnC with stretch accelerates the decline of the $[Ca^{2+}]_i$ transient without a change in its amplitude; our data (not shown

here[45]) confirm this prediction. It follows that Ca^{2+} release from TnC upon rapid unloading will increase at greater SL. The above hypothesis predicts, therefore, that the Ca^{2+} surge in the nonuniform muscle increases when the muscle operates at greater length; the increased Ca^{2+} surge may in turn increase SR Ca^{2+} release and accelerate propagation of the Ca^{2+} waves. Our findings illustrated in FIGURE 6 indeed show that stretch increases the initial $[Ca^{2+}]_i$ transient ΔCw and Ca^{2+} waves accelerated in proportion to the increase of the initial $[Ca^{2+}]_i$ transient.

The Ca^{2+} surge and ensuing Ca^{2+} waves start during the rapid decline of force during the twitch, while the sarcomeres in the strong muscle segments relax and lengthen; this rapid force decline also coincides with rapid shortening of the sarcomeres in the stretched segment. We tested the contribution of the rapid shortening to the induction of TPCs by eliminating shortening in the weakened segment by stretch at varied time during relaxation of the muscle. We measured SL in the border zone (diameter $\sim$100 μM) of the segment exposed to a BDM-containing jet, which precluded simultaneous $[Ca^{2+}]_i$ measurement. Hence, conclusions about the behavior of $[Ca^{2+}]_i$ had to be derived from the observed force and must be considered with caution. The stretches indeed eliminated sarcomere shortening during relaxation (FIG. 7 middle trace of SL panel), but did not enhance twitch force significantly. Elimination of shortening during relaxation of the sarcomeres in the weak segment did not abolish TPCs as is illustrated in FIGURE 7. F_{TPC} was unaffected when compared to F_{TPC} of F_{TPC} without the stretch and reduced by 50% compared to the F_{TPC} when the muscle had been stretched early during the twitch; the latter reduction of F_{TPC} was proportional to the difference in twitch force at short SL compared to long SL. These observations suggest that the shortening of sarcomeres in the weakened segment of muscle does not affect the induction of the Ca^{2+} wave and are consistent with the assumption that the Ca^{2+} wave is initiated by a Ca^{2+} surge that originates from the myofilaments as a result of the rapid decline of force.

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