Axial Stretch of Rat Single Ventricular Cardiomyocytes Causes an Acute and Transient Increase in Ca\textsuperscript{2+} Spark Rate

Gentaro Iribe, Christopher W. Ward, Patrizia Camelliti, Christian Bollensdorff, Fleur Mason, Rebecca A.B. Burton, Alan Garny, Mary K. Morpew, Andreas Hoenger, W. Jonathan Lederer, Peter Kohl

Abstract—We investigate acute effects of axial stretch, applied by carbon fibers (CFs), on diastolic Ca\textsuperscript{2+} spark rate in rat isolated cardiomyocytes. CFs were attached either to both cell ends (to maximize the stretched region), or to the center and one end of the cell (to compare responses in stretched and nonstretched half-cells). Sarcomere length was increased by 8.01 ± 0.94% in the stretched cell fraction, and time series of XY confocal images were recorded to monitor diastolic Ca\textsuperscript{2+} spark frequency and dynamics. Whole-cell stretch causes an acute increase of Ca\textsuperscript{2+} spark rate (to 130.7 ± 6.4%) within 5 seconds, followed by a return to near background levels (to 104.4 ± 5.1%) within 1 minute of sustained distension. Spark rate increased only in the stretched cell region, without significant differences in spark amplitude, time to peak, and decay time constants of sparks in stretched and nonstretched areas. Block of stretch-activated ion channels (2 μmol/L GsMTx-4), perfusion with Na\textsuperscript{+}/Ca\textsuperscript{2+}-free solution, and block of nitric oxide synthesis (1 mmol/L L-NAME) all had no effect on the stretch-induced acute increase in Ca\textsuperscript{2+} spark rate. Conversely, interference with cytoskeletal integrity (2 hours of 10 μmol/L colchicine) abolished the response. Subsequent electron microscopic tomography confirmed the close approximation of microtubules with the T-tubular–sarcoplasmic reticulum complex (to within ~10\textsuperscript{8}m). In conclusion, axial stretch of rat cardiomyocytes acutely and transiently increases sarcoplasmic reticulum Ca\textsuperscript{2+} spark rate via a mechanism that is independent of sarcolemmal stretch-activated ion channels, nitric oxide synthesis, or availability of extracellular calcium but that requires cytoskeletal integrity. The potential of microtubule-mediated modulation of ryanodine receptor function warrants further investigation. (Circ Res. 2009;104:787-795.)

Key Words: mechanolectric feedback ■ ryanodine receptor ■ stretch-activated channel ■ nitric oxide ■ electron microscopic tomography

Cardiomyocyte Ca\textsuperscript{2+} handling underlies the mechanical activity of the heart.\textsuperscript{1} In turn, cardiac Ca\textsuperscript{2+} handling is affected by the mechanical environment.\textsuperscript{2} In situ, cardiomyocytes are exposed to dynamically changing mechanical conditions, making it difficult to dissociate the bidirectional interplay of Ca\textsuperscript{2+} handling and mechanics. To obtain a better understanding of the dynamic integration of cardiac function, it is helpful to characterize in detail how the mechanical environment may affect individual facets of Ca\textsuperscript{2+} handling in intact isolated cardiomyocytes, such as spontaneous diastolic Ca\textsuperscript{2+} spark generation.

The sarcoplasmic reticulum (SR) is the major functional Ca\textsuperscript{2+} store in cardiac myocytes with important roles in cardiac excitation-contraction coupling. Its Ca\textsuperscript{2+} content is a key determinant of SR Ca\textsuperscript{2+} release.\textsuperscript{3} In resting cells, SR Ca\textsuperscript{2+} content ([Ca\textsuperscript{2+}]\textsubscript{SR}) is determined by the balance between Ca\textsuperscript{2+} uptake (via the sarco-/endoplasmic reticulum Ca\textsuperscript{2+} ATPase) and Ca\textsuperscript{2+} leak (largely in the form of Ca\textsuperscript{2+} sparks; release events via type 2 ryanodine receptors [RyR2\textsuperscript{2}]). This diastolic Ca\textsuperscript{2+} balance may be negative ("rest-decay phenomenon") or positive ("postrest potentiation"), depending on species. Rest decay is observed when the diastolic Ca\textsuperscript{2+} leak from the SR exceeds reuptake, such as in cat, guinea pig, rabbit, or frog.\textsuperscript{5} In other species, such as humans, mouse, and rat, postrest potentiation occurs.\textsuperscript{6,7} Interestingly, stretch appears to reduce [Ca\textsuperscript{2+}]\textsubscript{SR} compared with resting preparations at shorter length in species showing either type of overall diastolic Ca\textsuperscript{2+} balance.\textsuperscript{8,9}

Given their maintained high diastolic SR Ca\textsuperscript{2+} levels, rat cells have become a key experimental model for the study of...
diastolic SR Ca\(^{2+}\) leak via observation of Ca\(^{2+}\) sparks.\(^{10,11}\) Single-cell studies into related phenomena have, by and large, been conducted in mechanically unloaded cells, so that the effects of axial stretch on SR Ca\(^{2+}\) handling are generally not well elucidated. One prior report indicated that axial stretch reduces overall SR Ca\(^{2+}\) load in guinea pig cardiomyocytes within seconds of stretch application, but individual release events (sparks) were not studied.\(^9\) Another study showed that stretch increases Ca\(^{2+}\) spark rate in rat cardiomyocytes via a nitric oxide (NO)-mediated pathway, but this was observed after prolonged exposure to the mechanical stimulus (10 minutes).\(^{12}\)

In the present study, we investigated the acute effects on Ca\(^{2+}\) spark rate of diastolic stretch, applied axially to single intact rat ventricular cardiomyocytes. We found that this causes an acute and transient increase in Ca\(^{2+}\) spark rate, without affecting the dynamic behavior of individual sparks, via a pathway that requires cytoskeletal integrity.

**Materials and Methods**

**Cell Preparation**

Experiments were conducted in accordance with the guidelines of relevant institutional animal care and ethics regulations, and in agreement with the UK Home Office Animals (Scientific Procedures) Act of 1986. Adult rats were terminally anesthetized by injection of pentobarbital (100 mg·kg\(^{-1}\)), followed by cardiac excision and enzymatic isolation of ventricular myocytes, as described elsewhere.\(^{13}\) Cardiomyocytes were stored in a normal Tyrode solution, containing (in mmol/L): NaCl 140, KCl 10, CaCl\(_2\) 1.8, MgCl\(_2\) 1, HEPES 5, glucose 11. Experiments were performed at room temperature (22°C).

**Axial Stretch Technique**

The CF technique used in this study has been described in detail elsewhere.\(^{14}\) In short, a pair of CFs was attached to a single isolated cardiomyocyte using two 3-axis miniature hydraulic manipulators (SM-28, Narishige, Tokyo, Japan), each mounted on separate computer-controlled piezoelectric translators (PZT; P-621.1CL, Physik Instrumente, Karlsruhe/Palmbach, Germany) of a custom-made railing system (IonOptix, Milton, Mass). Axial stretch was applied by PZT movement of CFs, graded to cause an increase in sarcomere length of \(\approx8\%\) in the stretched portion of the cell. Larger strains increased the likelihood of either CF detachment or mechanical induction of Ca\(^{2+}\) waves (see “excess stretch wave” movie in the online data supplement, available at http://circres.ahajournals.org), both of which were exclusion criteria. Sarcomere length changes were confirmed via fast Fourier transformation of striation patterns in the confocal images (Figure 1). The Table summarizes the resultant changes in sarcomere length during half-cell stretch application, reconfirming that the target increase in sarcomere length was achieved in the stretched portion of the cell only, whereas the nonstretched cell side remained unaffected.

**Whole-Cell Stretch Protocol**

To maximize the mechanically affected cell proportion, CFs were attached to each cell end (whole-cell stretch; Figure 1A). Ca\(^{2+}\) spark rate was compared during 5-second intervals, before application of stretch, immediately after onset of stretch, and at the end of 1 minute of stretch.

**Half-Cell Stretch Protocol**

One CF was attached to the center of the cell, and the other CF was attached to one end of the same cell. The central CF remained stationary, whereas the end-standing CF was used to apply stretch to half of the cell only, leaving the remainder of the cell relatively undisturbed (half-cell stretch; Figure 1B). Ca\(^{2+}\) sparks were counted in both the stretched and the nonstretched portion of the cell, for 5 seconds, immediately before and after application of stretch, and the percentage change in Ca\(^{2+}\) spark rate (“during stretch” divided by “prestretch” times 100) was assessed separately for each cell half.

**Ca\(^{2+}\) Spark Measurements**

Cells were loaded with Fluo-4 by 10 minutes of incubation with Fluo-4-acetoxyethyl-ester (Invitrogen, Carlsbad, Calif) and scanned using an argon ion laser beam for illumination at 488 nm. Emitted fluorescence was detected above 505 nm. XY confocal images were acquired every 20 to 30 ms in a time series using an LSM 510 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Automated analysis of images for Ca\(^{2+}\) spark locations was performed using custom routines (available from C.W.W. [ward@son.umaryland.edu]), written in Interactive Data Language (IDL version 6.2). Some core-processing routines from previous work\(^{15,16}\) were modified and used for this XY time (XYT)

<table>
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<tr>
<th>Table 1. Changes in Sarcomere Length During Half-Cell Stretch Application (see Fig 1B)</th>
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<tr>
<td><strong>Sarcomere Length ((\mu m))</strong></td>
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<tr>
<td>Nonstretched Half-Cell</td>
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<tr>
<td>Before Stretch</td>
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<td>1.874±0.024</td>
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The relative sarcomere length increase in the stretched half-cell is \(8.01\% \pm 0.94\%\), while there is no change in the nonstretched cell portion. Data are mean±SEM; n=10.
For each time point of the XYT series, a 5-frame running average was performed to create a parallel XYT (XYTp) image array. Following the manual identification of an area outside the cardiomyocyte to measure background fluorescence, a 4/H11003 boxcar filter was applied to each image. From this, the area containing the cardiomyocyte was empirically identified as being 1.5 SD greater than the background fluorescence, and the mean intensity (Fmean,tot) and SD of total fluorescence within the cardiomyocyte boundary were established. Potential spark locations were identified as contiguous pixel regions with an intensity of 2 SD above Fmean,tot.

The XYTp image array was then reprocessed, to calculate mean intensity (Fmean,net) and SD of the net fluorescence in cardiomyocyte area outside potential spark locations. Thereafter, a ∆F representation (local fluorescence intensity minus Fmean,net) was constructed of each image, and Ca2+ sparks were finally confirmed as contiguous pixel areas with a local intensity that exceeded Fmean,net by 3.8 SD.10 Ca2+ spark frequency was analyzed, and, to obtain actual Ca2+ spark rates, duplicate counts of sparks at any coordinate (ie, those that lasted throughout more than one of the contiguous frames) were subtracted.

In addition, fast XYT series image acquisition (one 512×30 pixel frame captured every 1.5 to 2.5 ms) was performed during the half-cell stretch protocol, using an LSM 5-Live microscope to analyze spark dynamics. XY regions containing individual sparks (Figure 2A) were collapsed onto the x axis, to provide a 1D signal intensity line (pseudo line-scan image), and then all 1D pseudo line-scan traces were stacked in chronological order to create a 2D XT-sequence (Figure 2B, pseudo line-scan time plot). The time course of the signal at the center line of Figure 2B was then used to analyze spark amplitude, time to peak, and decay time constant of the spark (Figure 2C), also shown in a pseudocolor 3D surface plot (Figure 2D).

Electron Microscopy and Tomography
Spatial interrelation of microtubules with the T-tubular–SR membrane system that contains RyR2 was further investigated by electron tomography (ET). The principle of ET has been described in detail elsewhere.17 In short, adult rat ventricular cardiomyocytes were fixed in PBS containing 2% glutaraldehyde for 40 minutes and postfixed in 1% OsO4 for 10 minutes. Cells were then dehydrated in acetone and embedded in Epon-Araldite resin (Electron Microscopy Sciences, Hatfield, Pa). Sections (250 nm) were cut and transferred onto ET grids. Colloidal gold particles (15 nm) were added to both surfaces of the sections as fiducial markers for use during subsequent image stack alignment. Preparations were imaged with a Tecnai TF30 microscope operating at 300 kV (FEI Company, Eindhoven, The Netherlands), with images captured on an Ultrascan 4K CCD camera (GATAN Inc, Pleasanton, Calif). At the nominal magnification of 23 000, projected image dimension is 1.02×1.02 nm2 per camera pixel, providing a Nyquist resolution of 2.04 nm in the XY plane. Resolution in the Z direction of the sample is affected by the highest possible tilt angle α in the tomogram and cannot be better than the [XY resolution]×[sin(αmax)]−1 (for a detailed discussion of the technique and its limitations, see Lucic et al18). This theoretical maximum resolution, however, does not take into account the effects of cell isolation and fixation on subcellular structural integrity, or the challenges involved in distinguishing biological detail in densely populated cells such as cardiomyocytes. Physical resolution is
therefore not to be confused with the ability to resolve and properly interpret biologically relevant detail, which, here, is in the order of 4 to 5 nm. With this technique, it was possible to track with confidence the T-tubular–SR membrane system in the 3D ET data sets, as well as continuous microtubular structures.

For dual-axis tilt series imaging, the specimen holder was tilted from \( /H_1^11001 60^\circ \) to \( /H_1^11002 60^\circ \) at 1° intervals (121 images collected); the specimen was then rotated by 90° in the XY plane of the holder, and another \( /H_1^11001 60^\circ \) to \( /H_1^11002 60^\circ \) tilt series was taken. The images from each tilt-series were aligned (by fiducial marker tracking) and back-projected to generate 2 single full-thickness reconstructed volumes (tomograms), which were then combined to generate a single high-resolution 3D reconstruction of the original partial cell volume. Tomograms were processed using the IMOD software\(^9\) to generate 3D models of the relevant structures of interest. Microtubules were modeled as tubes with a diameter of 24 nm (shown in green in relevant figures and animations), whereas SR and T-tubular membranes were modeled by red and yellow contours (respectively) along the bilayer projection delimiting these distinct compartments, traced for each tomographic slice (see “3D-EM-tomography” movie in the online data supplement). The model was smoothed and meshed to obtain the final 3D representation, where spatial relationships among microtubules, SR, and T-tubules were analyzed.

Statistics
All values are presented as means±standard error of means. Paired Student \( t \) test and 2-way ANOVA were used for statistical assessment, where appropriate. A probability value of less than 0.05 was considered to indicate a significant difference between means.

Results
Figure 3 summarizes the result of stretch-induced changes in \( {\text{Ca}}^{2+} \) spark rate with the whole-cell stretch protocol. Data are normalized to \( {\text{Ca}}^{2+} \) spark rate in the same cell just before stretch application. Axial stretch caused an acute increase in \( {\text{Ca}}^{2+} \) spark rate to 130.7\( \pm \)6.4% of control (n=8, \( P<0.01 \)), followed by return to background levels (104.4\( \pm \)5.1%) within 1 minute of maintained diastolic distension (see “whole-cell-stretch” movie in the online data supplement).

Figure 4 illustrates an example of the stretch-induced increase in \( {\text{Ca}}^{2+} \) spark rate observed during application of half-cell stretch to a ventricular cardiomyocyte. Quantitative results are summarized in Figure 5A. Data from both the stretched and nonstretched cell half are normalized to the \( {\text{Ca}}^{2+} \) spark rate in the corresponding cell area before application of stretch. \( {\text{Ca}}^{2+} \) spark rate increased to 128.2\( \pm \)7.2% (n=10, \( P<0.01 \)) in the stretched part of the cell, whereas in the nonstretched half, it was not statistically different from Control (-5 – 0 s) Stretch (0 – 5 s) Stretch (60 – 65 s) Normalized spark rate (%) 0 20 40 60 80 100 120 140 160 Figure 3. \( {\text{Ca}}^{2+} \) spark rates observed with the whole-cell stretch protocol. \( {\text{Ca}}^{2+} \) spark rate increases immediately (within the first 5-second bin) after application of about 8% axial stretch, and returns to control levels (within 1 minute) in the presence of maintained stretch. Data are normalized to spark rate in the same cell before stretch application. \( * \)\( P<0.05 \) vs control.

Figure 4. Time course of relative Fluo-4 signal intensity in a rat ventricular resting cardiomyocyte, illustrating dynamic changes in spatially resolved \( {\text{Ca}}^{2+} \) concentration before and during half-cell stretch. The panel at the back shows an image of the cell, averaged from 10 confocal XY scans, before stretch application. Low signal intensity areas, overlapping the cell body, reveal CF positions before stretch (scale bar=20 \( \mu \)m). For the front, fluorescence intensity in each confocal XY scan was added along the \( y \) axis and plotted as a pseudo-3D XT sequence of relative calcium fluorescence (note sarcomere-related modulation of fluorescence intensity). Stretch was applied at 0 second by lateral movement of the CF near the cell end (right) (shading across XT plot indicates period of CF movement), stretching sarcomeres in the affected area by \( \approx \)8%, whereas sarcomere length in the unaffected cell region (left cell half) remains unchanged.
control (91.1±6.7%; see “half-cell-stretch” movie in the online data supplement).

Stretch affected neither spark amplitude (ΔF/F₀=0.50±0.03 in stretched versus 0.48±0.03 in nonstretched half-cell, n=16), nor time to peak (8.8±0.7 ms in stretched versus 8.5±0.5 ms in nonstretched half-cell, n=16) and decay time constant (18.2±1.4 ms in stretched versus 21.6±1.7 ms in nonstretched half-cell, n=16); all parameters assessed in sparks obtained during synchronous recording of stretched and nonstretched segments of the same cell (Figure 6).

Ca²⁺ spark rate may be augmented by extracellular Ca²⁺ influx causing Ca²⁺-induced Ca²⁺ release via RyR2. To investigate a possible involvement of extracellular Ca²⁺ influx via stretch-activated ion channels (SAC), we per-

**Figure 5.** Ca²⁺ spark rate during the first 5 seconds of stretch, applied to one half of the cell only. Data for the stretched and the nonstretched cell half are normalized to spark rates in the same area during the 5 seconds before stretch application. Responses in control conditions (A) and after treatment of cells with the following compounds are shown: 2 µmol/L GsMTx-4 (B); Na⁺/Ca²⁺-free solution (C), 1 mmol/L L-NAME (D), 10 µmol/L colchicine (E). Stretch caused a significant increase in Ca²⁺ spark rate in the stretched part of the cell in all experimental conditions, except after preincubation (for 2 hours) with colchicine. Number of observations in brackets; *P<0.05 (no significant differences were observed under any experimental conditions in the nonstretched cell region between spark rate before and after application of stretch to the other cell half).

**Figure 6.** Parameters describing Ca²⁺ spark dynamics during application of half-cell stretch. No significant differences were observed between sparks in the stretched and nonstretched cell areas in relative (F/F₀) spark amplitude (A), time to peak (B), and decay time constant (C). Number of observations shown in brackets; ns indicates nonsignificant.
formed the half-cell stretch protocol after 10 minutes preincubation with 2 μmol/L of the SAC blocker GsMTx-4. As illustrated in Figure 5B, GsMTx-4 did not affect the acute stretch-induced increase of Ca\(^{2+}\) spark rate (126.2±3.7% in stretched versus 91.5±10.1% in nonstretched half-cell; \(P=0.012\) and \(P>0.05\), respectively; \(n=9\)).

Because mechanically induced Ca\(^{2+}\) entry into cardiomyocytes could occur via other channels or transporters,\(^{20,21}\) we assessed possible roles of trans-sarcolemmal Ca\(^{2+}\) influx using the half-cell stretch protocol in Na\(^+/Ca\(^{2+}\)\)-free solution (ONC; containing [in mmol/L] LiCl 140, KCl 10, EGTA 10, MgCl\(_2\) 1, HEPES 5, glucose 11). The perfusate was switched from normal Tyrode to ONC 5 seconds before stretch application to record control spark rate. As shown in Figure 5C, the ONC environment did not abolish the acute stretch-induced increase in Ca\(^{2+}\) spark rate (131.7±8.4% in stretched versus 74.3±11.2% in nonstretched half-cell; \(P<0.01\) and \(P>0.05\), respectively; \(n=8\)).

To probe a possible involvement of NO in the acute response of Ca\(^{2+}\) spark rate to stretch, we performed the half-cell stretch protocol after 10 minutes preincubation with 1 mmol/L N\(^{\text{4}-}\)nitro-L-arginine methyl ester (L-NAME) to block NO synthase. As shown in Figure 5D, this intervention did not abolish the increase in Ca\(^{2+}\) spark rate in the stretched part of the cell (126.6±7.3% in stretched versus 96.5±7.0% in nonstretched half-cell; \(P<0.01\) and \(P>0.05\), respectively; \(n=7\)).

The possible involvement of cytoskeletal structures in the transmission of mechanical cues from cell surface to the T-tubular–SR complex was assessed by preincubation of cells, for 2 hours, with 10 μmol/L colchicine, which prevents microtubule polymerization. This protocol has previously been shown to cause a significant reduction in microtubular integrity in rat cardiomyocytes, reducing β-tubulin–specific fluorescence by 38% to 43%.\(^{22,23}\) Colchicine did not affect the control spark rate before stretch application (12 383±553 sparks · mm\(^{-2}\) · sec\(^{-1}\) in control, \(n=10\); versus 12 600±852 sparks · mm\(^{-2}\) · sec\(^{-1}\) in colchicine-treated cells, \(n=12\)), suggesting that nonspecific drug effects had little impact, if any, on the observed parameter. As shown in Figure 5E, after colchicine pretreatment no significant changes in Ca\(^{2+}\) spark rate were observed on acute axial distension (108.9±3.5% in stretched versus 99.0±5.2% in nonstretched half-cell, \(n=12\)).

The spatial interrelation of microtubules with the T-tubular–SR membrane complex was further investigated in 3D, using ET. Figure 7 shows 2 representative XY tomographic sections (taken in the plane of the preparation, 14.25 nm apart; Figure 7A and 7B). These reconstructed sections, which are qualitatively similar to the kind of data obtained in transmission ET, highlight the relative ease with which one can identify extended membrane structures (such as the T-tubular–SR membrane complex, highlighted in yellow and red, respectively, in Figure 7C), compared to the difficulty of tracking filamentous structures that proceed at an oblique angle relative to the imaging plane (such as microtubules). One advantage of ET is that one can “cut” the 3D imaging data set in any desired plane, for example coaligned with a microtubule of interest (Figure 7D). In addition, the ability to track membranes and filamentous structures throughout a physically connected 3D space allows one to reconstruct spatially accurate 3D models of microtubules, SR, and T-tubular membranes (Figure 7E). Microtubules regularly traverse the T-tubular–SR membrane complex, which contains the cytoplasmic domain of RyR2 (arrowheads in Figure 7C). In the studied examples, microtubules approach SR and T-tubular membranes to within 7 and 13 nm, respectively, suggesting spatial proximity that is close enough to support mechanical interaction.

**Discussion**

Diastolic stretch gives rise to an acute and transient increase in Ca\(^{2+}\) spark rate, without changing the dynamic properties of individual sparks. Using the whole-cell stretch protocol (which has the advantage of maximizing the observation area), it was established that the stretch-induced increase in Ca\(^{2+}\) spark rate (to 130.7±6.4% of control) occurs acutely, within the first 5 seconds (minimum observation period with sufficient power to conduct statistical analysis), and that this increase is of a transient nature, with spark rate returning to near control levels (104.4±5.1%) within 1 minute. This response cannot be explained by spatial rearrangement of RyR2, even though constant volume behavior predicts radial compression (by approximately −4%) during axial strain (by +8%). Because the total number of RyR2 in the stretched cell portion does not change either, net density of RyR2 in any subvolume that is large compared to the dimensions of a single sarcomere is unlikely to show a significant change. In addition, the transient nature of the observed spark rate response (in the presence of maintained stretch) makes it unlikely that spatial rearrangement of RyR2 plays a significant role in this context.

A possible explanation for the initial increase in spark rate would be that axial cell stretch causes membrane depolarization, promoting Ca\(^{2+}\) influx that could stimulate sparks. However, the local nature of the stretch-induced increase in Ca\(^{2+}\) spark rate, established in half-cell stretch experiments (Figures 4 and 5), argues against any mechanism of inherently whole-cell nature.

Alternatively, stretch could increase transsarcolemmal Ca\(^{2+}\) influx, perhaps via SAC, to an extent that might be small enough to have no effect on the membrane potential, while still acting locally to promote SR Ca\(^{2+}\) release events. However, the lack of an effect of either GsMTx-4 exposure (Figure 5B) or perfusion with ONC solution (Figure 5C) suggests that any contribution of trans-sarcolemmal Ca\(^{2+}\) fluxes to the acute stretch-induced increase in Ca\(^{2+}\) spark rate must be negligible.

This may be different during sustained axial stretch where, as previously illustrated by Gannier et al, an increase in resting [Ca\(^{2+}\)], may occur via a streptomycin-sensitive mechanism (streptomycin also blocks SAC\(^{2+}\)), perhaps involving Ca\(^{2+}\) influx via SAC, or secondary effects of Na\(^+\) influx via SAC on the Na\(^+/Ca\(^{2+}\)\) exchanger activity.\(^{25,26}\) This may contribute to an involvement of SAC in the slow force response to stretch (which is accompanied by an increase in [Ca\(^{2+}\)], transients).\(^{27,28}\) However, these mechanisms appear to require time periods of 5 minutes or more to affect cell function. In contrast, the acute increase in Ca\(^{2+}\) spark rate
observed here occurs in resting cells, within the first 5 seconds of stretch application, and even in the absence of extracellular Ca\(^{2+}\).

Similar time constraints appear to apply to NO-mediated effects of stretch on Ca\(^{2+}\) spark rate, which have been reported on exposure of rat cardiomyocytes to 10 minutes stretch.\(^{12}\) The acute stretch-induced increase in Ca\(^{2+}\) spark rate observed here is of a transient nature (Figure 3) and involves different mechanisms, as it is not blocked by preincubation with L-NAME (Figure 5D). Our data show no changes in spark amplitude, time to peak, or decay time constant (Figure 6). This suggests that stretch is unlikely to act via an increase in the Ca\(^{2+}\) conductance of individual RyR2 or in the number of RyR2 recruited in a spark event cluster.

An alternative explanation is that axial cell stretch, via a hitherto unidentified pathway, increases the open-probability of RyR2. This could cause an acute increase in Ca\(^{2+}\) spark rate, enhancing SR Ca\(^{2+}\) leak, and thereby causing partial depletion of the SR (as Ca\(^{2+}\), released into the dyadic cleft, will only partially be pumped back into SR, and partially extruded from the cell via Na\(^+\)/Ca\(^{2+}\) exchanger). Such early stretch-induced reduction in \([\text{Ca}\(^{2+}\)]_{\text{SR}}\) has been reported before.\(^{8,9}\) Because \([\text{Ca}\(^{2+}\)]_{\text{SR}}\) is an important driver of Ca\(^{2+}\) spark rate,\(^{4}\) this would allow the cell to return to near-equilibrium Ca\(^{2+}\) spark rates, once the opposing effects of stretch and SR Ca\(^{2+}\) load on RyR2 open probability have balanced out.

Several studies have discussed an involvement of the cytoskeleton in Ca\(^{2+}\) handling, partially with contradictory results.\(^{22,23,29}\) Most recently, cardiomyocytes from the murine model of Duchene’s muscular dystrophy (ie, the dystrophin null MDX mouse) have been reported to respond to increased mechanical loads (whether applied as axial stretch by CFS\(^{30}\) or via osmotic swelling\(^{31}\)) with an augmentation in SR Ca\(^{2+}\) release. Of particular interest, in this context, is the observation that among the compensatory adaptations in the MDX heart there is an \(\approx 1.4\)-fold increase in \(\beta\)-tubulin,\(^{32}\) which, based on our findings, may strongly contribute to mechanically-promoted SR Ca\(^{2+}\) release in this disease model.
Our findings highlight that microtubule integrity is obligatory for the acute stretch-induced increase in Ca\textsuperscript{2+} spark rate (Figure 5E). The actual mechanisms underlying this involvement of the cytoskeleton are not clear. Based on the close proximity of microtubules with SR and T-tubular membranes (10\textsuperscript{−8} m; Figure 7), one might speculate on the possibility of physical transmission of stress or strain from sarcolemmal CF attachment points to RyR2 or membrane areas near RyR2 via microtubules. As major force-bearing components of the nonsarcomeric cytoskeleton, microtubules contribute to cardiomyocyte stiffness during axial compression (when microtubules “buckle,” contributing to passive load and cell recoil), although they appear not to affect tensile or viscoelastic behavior during axial elongation (which is best explained by their ability to translocate in the direction of positive strain).\textsuperscript{33–36} Microtubules are laterally enforced, both by the cytosolic viscosity and by direct elastic cytoskeletal links, as can be illustrated by the observation that neighboring microtubules in cardiomyocytes “often buckle . . . in a coordinated manner, both temporally and spatially in phase.”\textsuperscript{37} Such coordinated buckling of microtubules in cardiomyocytes has been observed over distances in the 10\textsuperscript{−6} m region, highlighting the plausibility of mechanical interference between microtubules and the T-tubular–SR membrane complex.

It is possible, therefore, that microtubules mechanically interfere with the SR in a way that may affect RyR2 open probability in a manner akin to SAC activation. The approximately 10\textsuperscript{2} RyR2 receptors within the Ca\textsuperscript{2+} release unit are thought to interact with each other mechanically, contributing to the coordinated generation and termination of Ca\textsuperscript{2+} sparks,\textsuperscript{38} and it is only a modest extension of this notion to suggest that RyR2 gating may be mechanically influenced by microtubule-mediated perturbation of the T-tubular–SR membrane complex. Similar deformation-induced increases in Ca\textsuperscript{2+} spark rate have been observed in the depth of atrial myocytes during sarcolemmal fluid-jet stimulation.\textsuperscript{39} Also, RyR2 mechanosensitivity could underlie the fluid-pressure induced increase in Ca\textsuperscript{2+}−induced Ca\textsuperscript{2+} releasability from the SR, observed in rat ventricular cardiomyocytes.\textsuperscript{40} Alternatively, there may be mitochondria-mediated responses,\textsuperscript{39} or currently unknown effects of fast-acting local signal transduction pathways that are relevant for RyR2 function and affected by the cytoskeleton.\textsuperscript{41}

In conclusion, axial stretch of rat cardiomyocytes acutely and transiently increases Ca\textsuperscript{2+} spark rate via pathways that are independent of SAC, NO, and transsarcolemmal Ca\textsuperscript{2+} influx but that do require cytoskeletal integrity. The mechanisms, interplay, and functional relevance of acute and late stretch effects on Ca\textsuperscript{2+} spark rate, as well as the interrelation of cytoskeletal elements with Ca\textsuperscript{2+} handling cell structures, form worthwhile targets for further elucidation.

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Disclosures
None.

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Axial Stretch of Rat Single Ventricular Cardiomyocytes Causes an Acute and Transient Increase in Ca\(^{2+}\) Spark Rate

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Supplement Material:

Movie 1 ‘whole-cell-stretch’: Illustration of the acute effect of axial cardiomyocyte elongation, applied using carbon fibres attached to both ends of a rat intact isolated cardiomyocyte, on Ca$^{2+}$ spark activity, assessed by monitoring Fluo-4 signal intensity. Stretch of the cell (sarcomere length increase by ~8%) gives rise to an acute increase in spark rate to about 130% of control. This is followed by return to background levels (within 1min of maintained diastolic distension; not shown).

Movie 2 ‘half-cell-stretch’: Illustration of the local nature of the acute stretch-induced increase in Ca$^{2+}$ spark rate, revealed by application of axial cardiomyocyte elongation to part of the isolated cardiomyocyte only.

Movie 3 ‘excess-stretch-wave’: Carbon fibre translocation was gauged to cause an increase in sarcomere length of about 8%. Larger strain levels were associated with an increased risk of carbon fibre detachment or initiation of spontaneous calcium waves, as shown here.

Movie 4 ‘3D-EM-tomography’: The utility of electron microscopy (EM) based tomography is illustrated by panning through consecutive XY EM sectioning planes of the data set, from ‘bottom’ to ‘top’ of the sample. The first frame is frozen to indicate the location of T-tubular membrane (yellow), sarcoplasmic reticulum (red) and a nearby microtubule (green). This is followed by stripping away the processed cell volume (from ‘top’ to ‘bottom’), exposing the
3D reconstructed model of the spatial interrelation of T-tubular—SR membrane system and microtubule.

Note: movies 3 and 4 are coded in MPG4 format; you may need to download a suitable player, such as VLC Media Player, freely available at http://www.videolan.org/vlc/.