An increase in force after stretch of diaphragm fibers and myofibrils is accompanied by an increase in sarcomere length non-uniformities

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An increase in force after stretch of diaphragm fibers and myofibrils is accompanied by an increase in sarcomere length non-uniformities and Ca$^{2+}$ sensitivity

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When muscle fibers from limb muscles are stretched while activated, the force increases to a steady-state level that is higher than that produced during isometric contractions at a corresponding sarcomere length, a phenomenon known as residual force enhancement (RFE). The mechanisms responsible for the RFE are an increased stiffness of titin molecules which may lead to an increased Ca\(^{2+}\) sensitivity of the contractile apparatus, and the development of sarcomere length non-uniformities. RFE is not observed in cardiac muscles, which makes this phenomenon specific to certain preparations. The aim of this study was to investigate if the RFE is present in the diaphragm, and its potential association with an increased Ca\(^{2+}\) sensitivity and the development of sarcomere length non-uniformities. We used two preparations: single intact fibers and myofibrils isolated from the diaphragm from mice. We investigated RFE in a variety of lengths across the force-length relationship. RFE was observed in both preparations at all lengths investigated, and was larger with increasing magnitudes of stretch. RFE was accompanied by an increased Ca\(^{2+}\) sensitivity as shown by a change in the force-pCa\(^{2+}\)-curve, and increased sarcomere length non-uniformities. Therefore, RFE is a phenomenon commonly observed in skeletal muscles, with mechanisms that are similar across preparations.
INTRODUCTION

When a muscle fiber is stretched while activated, the force increases and stabilizes at a level that is higher than that produced during isometric contractions developed at a corresponding sarcomere length. This residual force enhancement (RFE) is present in skeletal muscle fibers (16, 22, 36), myofibrils (11, 21, 50), sarcomeres (48) and half-sarcomeres (35), with increases in force of up to ~28-30%. However, RFE is not observed in cardiac muscles; well-controlled studies with isolated myofibrils from either ventricle (11) or papillary muscles (50) have failed to find an enhancement in force after stretch, a result that was confirmed in a study using the whole, intact cardiac trabeculae (57). This difference has been interpreted in light of a mechanism of the RFE; an increase in the stiffness of titin induced by activation and stretch – referred to as static tension (11, 50, 57). While the static tension is present in skeletal muscles, it is not observed in cardiac muscles (11), likely due to different physiological demands. It is now clear that the RFE is caused by a combination of the static stiffness and sarcomere length non-uniformities that happen during activation and stretch (15, 21, 48), such that some sarcomeres will have a larger than anticipated overlap and active force production, while others will increase their passive force significantly (14). Additionally, the static stiffness – non-related to cross-bridges – has been associated with an increase in the stiffness of the elastic protein titin, which increase with the presence of $\text{Ca}^{2+}$ (27) and during activation of skeletal muscles, but not cardiac muscles (11, 12).

When comparing striated muscles, there is still a gap in the literature; there are no studies looking into the presence and potential mechanisms of RFE in the diaphragm muscle. The diaphragm has characteristics that are unique: it is a respiratory muscle that contracts rhythmically against elastic and resistive forces, such that in each relaxation the diaphragm returns to a relatively constant length at a resting position, determined by the balance of lung and chest wall recoil forces [e.g., (9)]. Limb muscles also contract rhythmically during locomotion, but they must overcome predominantly inertial forces. The most prominent feature of the diaphragm is the fact that, like the heart, it must contract in a repetitive rhythm during a lifetime. Although the differences between elastic, resistive and inertial forces cannot likely explain differences in RFE, they still provide the diaphragm with unique contractile characteristics.

In this study, we evaluated the potential presence and mechanism of RFE in the diaphragm. We took advantage of two unique preparations from our laboratories that allow the evaluation of the cellular and sub-cellular mechanisms of RFE: intact living fibers and isolated myofibrils from the
mice. Intact fibers permit the evaluation of muscle mechanisms using the smallest contractile system that still maintains its main physiological characteristics (1, 4, 10, 37, 47); the fibers can be activated by electrical stimulation, which initiates action potentials, and can be experimented for many contractions. Myofibrils allow the control of the media surrounding the preparations while rapidly activating and relaxing the contractile system, the direct measurement of individual sarcomere lengths, and a precise measurement of specific force given its small cross-sectional area (~1 μm) (21, 41, 50, 51). We observed RFE in the diaphragm, which was accompanied by an increase of sarcomere length non-uniformities and myofibrillar Ca\(^{2+}\) sensitivity of the contractile apparatus. The result lead us to develop a stochastic model to understand how sarcomere length non-uniformities and an increase in the stiffness of titin in a myofibril can lead to RFE. Using this model, we were able to reproduce our experimental observations.

METHODS

In this study, we used small bundles with intact diaphragm fibers and isolated diaphragm myofibrils from the mouse. The animal protocol for the work with mice intact fibers was undertaken in compliance with the guidelines of the European Communities Council Directive 2010/63/UE and the recommendations for the care and use of laboratory animals and it was approved by the animal care Committee of the University of Florence (Italy) and Italian health Ministry (Authorization 708/2017-PR). The animal protocol for the mice myofibrils was approved by the Animal Care Committee at McGill University and the Canadian Council on Animal Care (Reference number: 20122–23).

Intact fiber preparation.

Experiments with intact fibers were performed using CD1 mice (Envigo, RMS Srl, Udine, Italy). Animals were housed with controlled temperature (21–24 °C) and a 12–12 h light-dark cycle. Food and water were provided ad libitum. Female mice (7–8 months old) were killed by prompt cervical dislocation to minimize animal suffering. The diaphragm muscle was quickly excised and placed in Tyrode solution with the following composition (mM): NaCl, 121; KCl, 5; CaCl\(_2\), 1.8; MgCl\(_2\), 0.5; NaH\(_2\)PO\(_4\), 0.4; NaHCO\(_3\), 24; glucose, 5.5; EDTA, 0.1 and bubbled with 5% CO\(_2\)–95% O\(_2\) which gave a pH of 7.4. Small bundles were dissected from the diaphragm as described previously (Ref. 1,2 and 3). The dissection was performed manually under a stereomicroscope with a fine pair of scissors taking care to avoid stretching and to obtain preparations clean of debris from dead fibers.
Small aluminum T-shaped clips were fixed to the tendons as close as possible to the fiber ends. The fibers were transferred to a temperature controlled experimental chamber (801C/1900, Aurora Scientific, Toronto CA) and mounted on an inverted microscope (Olympus IX71). The clips attached the fibers horizontally between the lever arms of a capacitance force transducer (405A, Aurora Scientific) and a length controller (322 Aurora Scientific). The fibers were superfused continuously with oxygenated Tyrode solution by a peristaltic pump. The experiments were performed at 27–28°C.

The resting bundle length, the bundle largest and smallest diameters were measured with a microscope fitted with 20× eyepieces and a 5× dry objective in the experimental chamber. The preparation length in our fibers (L_o, clip to clip) was 9.6 ± 0.68 mm (mean ± S.E.M, n = 15), of which 8.6 ± 0.66 mm was composed of the fibers and the remaining (0.99 ± 0.22 mm) was composed of tendons. The cross-sectional area of the bundles was calculated as a*b*π/4 where a and b are the average values of the width and the vertical height of the preparations respectively, measured at 2–3 different points along the bundles. The average cross-sectional area of the fiber bundles was 0.08 ± 0.01 mm^2.

**Experimental protocol.** After a 15 min equilibration period, bipolar stimuli (1 ms duration at 1.5 times threshold voltage) were applied across the fibers by two platinum-plate electrodes mounted parallel to the bundle via a high-power bipolar stimulator (701C Aurora Scientific). After testing the fibers viability, in which tetanic stimulation (1 sec duration) was applied at 1 min intervals using the minimum frequency necessary to obtain a fused contraction (100 Hz), the fibers were set at a length (L_o) at which tetanic force was maximal (P_o). In the current experiments, this length corresponded to a mean sarcomere length of ~2.70 μm. Tetani were given for a period of approximately 10 min; if P_o decreased by >15%, the fibers were discarded. Following the stabilization period, one of the two protocols was used:

(i) Bundles were subjected to two different series of contractions (3.5 sec in duration, elicited every 2 min). During the first protocol, starting from L_o, the bundles were stretched first to reach a new length the L_i length at which the tetanic tension produced was just lower than P_o, and then passively stretched by 4% and 8% of L_o to reach the final length L_f. At this length, L_f, a tetanic contraction was elicited. The entire sequence of stretches and contraction was repeated but only after a new L_i length was reached. Every time the new starting L_i length was a little bit longer and the corresponding developed tension a little bit lower than the previous one. It was estimated that we would
have a 6% decrement of tension between each pair of $L_i$ value. In this way for each bundle and for the different stretch amplitudes tested it was possible to obtain more than one point of measure, exploring the descending limb in terms of RFE as far as the saturation transducer permitted. Between contractions produced at different $L_i$, tetanic reference contractions were performed to check if the isometric maximal $P_0$ force produced at $L_o$ was constant throughout the experiments.

(ii) A second series of contractions was performed to evaluate the RFE. The duration of these contractions was sufficient to reach steady-state forces following stretch, i.e., when the force–time trace following active stretch was parallel to the force-time trace of the corresponding isometric contraction. In this series of contractions, starting from $L_i$, the bundle was stimulated and an active variable stretch, $L_{i+\%}$, was applied at the tetanic plateau.

The RFE was measured as the difference between the two traces $L_{i+\%}$ and $L_f$, 100 ms before the end of stimulation. Figure 1 shows an example from an experiment conducted to evaluate the RFE, with an isometric contraction and a contraction in which a stretch (4% $L_o$) was applied to the fiber. Passive and active stretches applied in amplitudes of 4% and 8% of $L_o$ (stretch durations of 0.2 sec and 0.4 sec, respectively).

In sum, one full stretch experiment consisted of the following tests: isometric contractions at $L_o$, $L_i$, and $L_f$; passive and active stretches starting from $L_i$. Following, a next stretch experiment at a new $L_i$ was performed. The whole experimental protocol induced a large number of contractions, but it is known that the maximal force developed by intact muscle bundles remains stable for many hours when the fibers are stimulated at regular intervals (2 min) with tetanic stimulation frequency (100 Hz) (2, 39).

The output of the force transducer and the length controller were acquired in real time using an integrated PC board (NI6221, National Instruments USA). The experimental data were displayed and successively analyzed with a dedicated controlled software (600A Digital controller, Aurora Scientific, CA).

Since (i) changes in fiber length in muscle bundles do not correspond exactly to changes in sarcomere length across experiments, and may lead to comparisons of contractions made at very
different lengths, and (ii) there is a large variability in the force-length relation obtained with
contractions developed using fixed-end experiments (i.e., when sarcomere length is not controlled
during the experiments), an estimation of length variations in the diaphragm bundles was derived
from the $L_i/P_0$ ratio – the force produced at $L_i$ relative to the force developed at $L_0$. In this way, we
guaranteed that we were comparing contractions produced when the isometric forces were similar
when compared to the forces produced at $L_0$, and therefore produced at comparable fiber lengths
assuming. All the RFE measures obtained during the experiments were grouped in five classes with
interval of 10% $L_i/P_0$. Before grouping, each value was expressed in terms of specific force
mN/mm$^2$. Then the average value of each class was plotted.

Myofibril preparations

Experiments with myofibrils were performed with CD1 mice (Charles River Canada). Small muscle
bundles of the diaphragm muscle were dissected, tied to wood sticks, and chemically permeabilized
following standard procedures used in our laboratory (44, 49-51). Muscles were incubated in rigor
solution (pH = 7.0) for approximately 4 hours, after which they were transferred to a rigor: glycerol
(50:50) solution for approximately 15 hours. The samples were subsequently placed in a fresh rigor:
glycerol (50:50) solution with the addition of a cocktail of protease inhibitors (Roche Diagnostics,
USA) and stored in a freezer ($-20\, ^\circ C$) for at least seven days. On the day of the experiments, small
pieces of the samples were homogenized following standard procedures, which resulted in a
solution containing isolated myofibrils. The myofibrils were transferred to the experimental setup,
which contained a system for detection of atomic force cantilever (AFC) displacements (29).

Measurements of myofibril forces. The myofibrils were attached between the AFC and a rigid
glass needle. A multichannel fluidic system connected to a double-barreled pipette was used for fast
activation and relaxation of the myofibrils. During the experiments, the position of the double-
barreled pipette was rapidly switched to change the solutions surrounding the myofibrils (pCa 4.5
and pCa 9.0; pCa = $-\log_{10}[Ca^{2+}]$, solutions described below). Such procedure allows for a fast
activation and relaxation of the myofibrils without damages to the preparation, and has been
explained in details in previous studies (44, 49-51). Under high magnification, the contrast between
the dark bands of myosin (A-bands) and the light bands of actin (I-bands) provided a dark-light
intensity pattern, representing the striation pattern produced by the sarcomeres, which allowed
measurements of sarcomere length during the experiments. The sarcomere length was measured
with a video camera connected to the right-side port of the microscope. Once the myofibrils were
attached between the AFC and the micro-needle, they were adjusted to an average sarcomere length of 2.7µm. Throughout the experiments, homogeneity of sarcomere length was assessed visually to detect potential damage in the myofibrils. When non-uniformity of sarcomere lengths formed during activation did not recover into a regular striation pattern before the next activation, the experiment was stopped and the myofibril was discarded from further analysis.

The myofibril length was more variable than the intact fiber length during the experiments, as myofibrils of distinct lengths were produced during the homogenization protocol and were selected based on their appearance; the length was 19.16 ± 5.31 µm (mean ± S.E.M, total n in all protocols used in this study = 23). The cross-sectional area of the myofibrils, calculated using a method similar to that used in intact fibers (see above) was 1.07 ± 0.14 µm.

Solutions: The rigor solution (pH 7.0) used to store the myofibrils was composed of (in mM): 50 Tris, 100 NaCl, 2 KCl, 2 MgCl₂, and 10 EGTA. The relaxing solution used for muscle dissection (pH 7.0) was composed of (in mM) 100 KCl, 2 EGTA, 20 imidazole, 4 ATP, and 7 MgCl₂. The experimental solutions used during the experiments (pH 7.0) was composed of (in mM) 20 imidazole, 14.5 creatine phosphate, 7 EGTA, 4 MgATP, 1 free Mg²⁺, free Ca²⁺ ranging from 1 nM (pCa 9.0) to 32 µM (pCa 4.5), and KCl to adjust the ionic strength to 180 mM. The final concentrations of each metal-ligand complex were calculated using a computer program based on previous studies (Ref). All experiments were performed at 25-27 °C.

**Force and sarcomere length calculations.** Force measurement using the AFC was performed as previously described (29, 49). Briefly a laser is shined upon and reflects from the AFC, which acts as a force transducer. When an attached myofibril is shortened due to activation it causes deflections of the AFC, which is detected and recorded using an optical system with a time resolution in the order of milliseconds. The force produced by a single sarcomere is equivalent to that of a single myofibril and was calculated using

\[ F(t) = kC \times c(t) \]  

where \( F(t) \) is the force per myofibril as a function of time \( t \), \( c(t) \) is the displacement of the cantilever tip (sampling rate: 0.2 MHz averaged over 1280 data points), and \( k_C \) is the stiffness of the silicon cantilever. The cantilever stiffness was determined either by measuring the cantilever dimensions through scanning electron microscope images, or by constructing force-bending curves.
using cantilevers previously calibrated with the scanning electron microscope images (50, 51). The sarcomere length of the myofibrils was calculated using

\[ S(t) = \frac{(M(0)-[c(t)-c(0)]+[n(t)-n(0))]}{SN} \]  

(2)

where \( S(t) \) represents the average sarcomere length as a function of time and captures the central tendency of the sarcomeres given any non-uniformity in the sarcomere length behaviour. Moreover, \( M(0) \) is the myofibril length at rest, \( c(0) \) is the initial position of the cantilever tip, \( n(t) \) is the input motion of the micro-needle, \( n(0) \) is the initial position of the micro-needle, and \( S_N \) is the number of sarcomeres within the myofibril.

The random errors in \( F(t) \) and \( S(t) \) were minimized in four ways. First, cantilever displacement was calibrated before the start of each experimental set using the laser-tracking system. Second, the micro-needle was at least 20 times thicker than the cantilever and did not deflect during the experiments. Third, the micro-needle was controlled by a piezoelectric controller with resolution greater than the noise of the system and did not limit the detectability of the myofibril response. Fourth, myofibrils with a large number of sarcomeres were studied so that the SL could better represent the central tendencies of the sarcomeres, despite any non-uniformity in behaviour.

**Experimental protocol.** RFE was measured using a protocol that was similar to that one used with intact fibers, with a few characteristic differences since we used the sarcomere length (instead of length changes as a percentage of \( L_o \)) to set the preparations through the experiments. The myofibrils (n = 12) were adjusted so that the nominal average sarcomere length was 2.7 μm (actual: 2.74 ± 0.15 μm, as verified after data analysis) before a contraction was induced by activation solution. After the onset of activation, contractions were held steady for 15 s before the surrounding solution was switched to the relaxing solution, which induced full relaxation. After two control contractions (3 min interval), the myofibrils were set sarcomere lengths of 2.9 μm, and 3.0 μm in random order, and isometric contractions were induced at these lengths. Finally, the myofibrils were set at lengths of 2.7 μm, 2.8 μm or 2.9 μm, and stretched to 2.9 μm or 3.0 μm (stretch magnitudes of approximately 4% or 8%, respectively). Stretches were induced at a speed of 0.3 μm/s during force production to reach a sarcomere length similar to the isometric contraction at \( L_f \). After stretch, an isometric contraction was repeated again to check for potential damage of the myofibril.
**Force-pCa$^{2+}$ relationship and Ca$^{2+}$ sensitivity of the contractile apparatus.** We performed additional experiments with myofibrils (n=9) to check for the Ca$^{2+}$ sensitivity of the contractile apparatus after stretch, and during the development of RFE. For these experiments, we first set the myofibrils at a sarcomere length of 2.9 μm and exposed them to different Ca$^{2+}$ concentrations (pCa$^{2+}$ of 7, 6.5, 6, 5.75, 5.5, 5, and 4.5). Then we performed experiments in which we stretched the myofibrils from 2.8 μm to 2.9 μm at the same Ca$^{2+}$ concentrations. The two force-pCa$^{2+}$ curves were fit to Hill's equation:

$$F = \frac{(Ca^{2+}/K)^{nH}[1 + (Ca^{2+}/K)^{nH}]}{1 + [1 - (Ca^{2+}/K)^{nH}]}$$

where $F$ is the normalized force, $K$ is the dissociation constant ($pK = -\log K = pCa50$), $nH$ is the Hill coefficient, and $F_{min}$ is the minimum force levels at a high pCa$^{2+}$. pCa50, corresponds to the half-maximal force response, an index that we have used previously as an indicator of Ca$^{2+}$ sensitivity (60).

**Sarcomere length dispersion.** During the experiments with myofibrils, it was possible to measure the length of individual sarcomeres throughout the experimental procedures. Such measures allowed us to evaluate the sarcomere length dispersion and non-uniformity during activation and stretch in the different experimental conditions. The dispersion for each myofibril was found by calculating the difference of individual sarcomere lengths to the average sarcomere length of one myofibril, as follows.

$$SL_{dispersion} = |SL_{individual} - SL_{average}|$$

**Statistical analysis.** Forces produced by the fibers and myofibrils in similar lengths during isometric contractions and stretch contractions were compared using a two-way analysis of variance (ANOVA) for repeated measures. Sarcomere length dispersion between rest, activation and after stretch was compared using a one-way ANOVA for repeated measures. The pCa$^{2+}_{50}$ obtained during isometric contractions or after stretch was compared using a one-way ANOVA for repeated measures. All post hoc analyses were performed with the Holm–Sidak test when needed (statistical package: SigmaPlot 13, Systat Software Inc., San Jose, CA, USA). A significance value of 5%
(P < 0.05) was adopted in all comparisons. Data are presented as mean ± S.E.M, or in box plots showing the also the interquartile ranges.

**Stochastic model of residual force enhancement.** Experimental results were reproduced in stochastic simulations of a myofibril.

**INSERT FIGURE 2 HERE**

These models were built up from the fundamental actomyosin interaction to the sarcomere levels, in which we were able to manipulate both sarcomere-non-uniformities and the changing stiffness of titin upon stretch. The model represents an extension of a previously described model (13), which is built upon the framework developed by Campbell (7). We build up our model of a myofibril starting with the individual force-dependent actomyosin chemomechanical cycle. We then model a half-sarcomere as numerous myosin motors in parallel with one another and with a passive elastic element representing titin. Finally, we develop our model of a myofibril by connecting numerous half-sarcomeres in series. The values of all constants described in the model are presented in Table 1.

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<th>Value</th>
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11
\[
\begin{align*}
\text{Table 1: Constants associated with the chemomechanical cycle of a cross-bridge and the mechanical properties of the cross-bridge and the non-linear passive spring element.}
\end{align*}
\]

Model of an individual cross-bridge.

The states which the actomyosin system cycles are depicted in Figure 2A, where State 0 is a weakly associated state between the actin and myosin, and States 1 and 2 are strongly associated stages, with the motor’s power stroke occurring during the transition from State 1 to State 2. The power stroke occurs over a distance \( d \), and we define the distance from State 1 to the transition state of the powerstroke transition to be \( \delta_1 \) and the distance from State 2 to this transition state to be \( \delta_{-2} \). We require \( d = \delta_1 + \delta_{-2} \)

As indicated in Figure 2A, all rates are modeled as force dependent except for the actin-attachment rates for the motor (\( k_0 \) and \( k_{-0} \)); the force dependent rates are described in detail below. The cross bridge is modeled as a Hookean spring with stiffness \( \sigma \), and so the force that an individual motor experiences at a given extension of its cross-bridge is given by

\[
F = -\sigma \epsilon
\]

where \( \epsilon \) is the extension of the linear spring in the positive direction (which we have defined as the direction in of the power stroke).

(A) Force dependent rates associated with the power stroke.

In the model, \( k_1(F) \) is the rate associated with the power stroke. Assuming that the motor is experiencing a force in the negative direction due to a positive value of \( \epsilon \), the motor must do work against this opposing force to reach the transition state of the power stroke. Because the power stroke itself changes \( \epsilon \), this opposing force is not constant. However, we can calculate the average force that the motor experiences as it approaches the transition state to be

\[
F_{\text{forward}} = -\kappa_{cb} \left( \epsilon + \frac{\delta_1}{2} \right) = F + \kappa_{cb} \frac{\delta_1}{2},
\]
where $\varepsilon$ is the extension of the cross bridge prior to the power stroke. We then model this force dependent rate using Bell’s model (6),

$$k_1(F) = k_1(0)\exp\left(\frac{F_{\text{forward}}\delta_1}{k_BT}\right).$$ (7)

By a similar argument, we model the force dependence of the rate $k_{-2}(F)$, which describes the transition associated with reversing the power stroke. In this case, the average force that the motor experiences as it approaches the transition state in the reverse direction is

$$F_{\text{reverse}} = -\kappa_{cb} \left(\varepsilon - \frac{\delta_2}{2}\right) = F - \kappa_{cb} \frac{\delta_2}{2}. \quad (8)$$

We again model this rate using Bell’s model,

$$k_{-2}(F) = k_{-2}(0)\exp\left(-\frac{F_{\text{reverse}}\delta_2}{k_BT}\right). \quad (9)$$

(B) Force dependent rates associated with the detachment of the motor from actin

We model the force dependence of the rates associated with detachment from actin ($k_{-1}(F)$ and $k_2(F)$) assuming a catch-slip mechanism for actomyosin detachment, as described by Guo et al. (19). According to this model, at low forces, the rate of detachment of the motor from actin decreases with force, while at larger forces, the rate increases. We describe this situation using two Bell-like terms, where the first term describes a rate that decreases with force and the second term describes a rate that increases with force:

$$k_2(F) = k_{2a}(0)\exp\left(-\frac{|F|\delta_{2a}}{k_BT}\right) + k_{2b}(0)\exp\left(|F|\delta_{2b}\right) \quad (10)$$

$$k_{-1}(F) = k_{-1a}(0)\exp\left(-\frac{|F|\delta_{-1a}}{k_BT}\right) + k_{-1b}(0)\exp\left(|F|\delta_{-1b}\right) \quad (11)$$

Model of a half-sarcomere.
To determine the number of motors that can potentially interact with the thin filament in an activated half-sarcomere, we calculate the overlap of the thick and thin filaments as

\[ x_{\text{overlap}} = l_{\text{thin}} + l_{\text{thick}} - x_{hs}, \]  

(12)

where \( x_{hs} \) is the length of the half-sarcomere, and the other parameters describe the geometry of a half-sarcomere, as depicted in Figure 2B.

The number of myosin heads per unit cross-sectional area in a single half-sarcomere framework is about \( N_o = 1.15 \times 10^{17} \) m\(^{-2}\) (7, 30), and the cross-sectional area of the myofibril is about \( A = 10^{-12} \) m\(^2\). The number of motors that can engage with the thin filament are calculated as

\[
N_{\text{motors}} = AN_o \left( \frac{x_{\text{overlap}}}{x_{\text{max,overlap}}} \right) \quad x_{\text{overlap}} < x_{\text{max,overlap}}
\]

(13)

\[
N_{\text{motors}} = AN_o \quad x_{\text{overlap}} > x_{\text{max,overlap}}
\]

To investigate the effects of heterogeneities in half-sarcomere lengths on RFE, we needed to introduce variability in the half-sarcomere lengths. To this end, we used the approach taken by Campbell (7), and assumed that the number of interacting myosin heads per half-sarcomere was a normally distributed variable with a variance that we could choose. To achieve this, we defined the actual number of interacting motors as

\[
N_{\text{interacting}} = G(\alpha) N_{\text{motors}}
\]

(15)

where \( G(\alpha) \) is a number randomly selected from a normal distribution with a mean of one and a variance of \( \alpha \). The values of \( \alpha \) used in our model are described below.

We modeled each of the interacting motors as being connected in parallel with one another and with a passive non-linear elastic element, representing titin. The force generated by stretching this non-linear element is given by
\( F_{\text{nonlinear}}(x_{hs}) = \sigma A \exp \left( \frac{x_{hs} - x_{\text{offset}}}{l} \right) \) \hspace{1cm} (16)

**Model of a myofibril.**

Myofibrils consist of \( N \) half-sarcomeres connected in series. The \( N + 1 \) positions of each boundary are given by \( y_j \), where \( j = 0, 1, 2 \ldots N \) (Figure 2C). The length of each half-sarcomere \( x_{hs,i} \) is given by the relation

\[ x_{hs,i} = y_{i+1} - y_i \] \hspace{1cm} (17)

where \( i = 0, 1, 2 \ldots N - 1 \).

**Stochastic simulations.**

Using the procedures described below, we compared three simulated conditions for myofibrils consisting of \( N = 50 \) half-sarcomeres:

A) Isometric activation of a myofibril at an average sarcomere length of 3 \( \mu \text{m} \) (average half-sarcomere length = 1.5 \( \mu \text{m} \));

B) Isometric activation of a myofibril at an average sarcomere length of 2.8 \( \mu \text{m} \) (average half-sarcomere length = 1.4 \( \mu \text{m} \)) followed by a stretch of 0.1 \( \mu \text{m}/\text{half-sarcomere} \) at a rate of 0.1 \( \mu \text{m}/\text{s/} \text{half-sarcomere} \);

C) The same process as (B), with the modification that, when the stretch is initiated, the stiffness of the non-linear element increases. This adjustment is meant to capture the observation that, following activation, stretch of titin results in an increased stiffness in this non-linear spring.

**(A) Isometric activation at half-sarcomere length of 1.5 \( \mu \text{m} \)**

**(i) Initial conditions.**

The boundaries of the \( N \) half-sarcomeres were set so that \( y_0 = 0 \) and \( x_{hs,i} = 1.5 \mu \text{m} \) for all values of \( i \). Because we assume that heterogeneities in half-sarcomere lengths arise as a result of stretch, throughout isometric activation, \( \propto = 0 \). Before activation, all motors start in State 0. Upon activation, the motor is allowed to evolve into other states according to the rates described in Table 1 and Equations 2-7, as described below. The simulation proceeds iteratively through steps (ii-iv), using a time step of \( \Delta t = 1 \text{ ms} \).
(ii) **Determine the number of interacting motors.**

We use the values of $y_j$ to determine the length of each half-sarcomere $x_{hs,i}$ using equation 17. Then, using the process described above, we calculate the number of interacting motors for each half-sarcomere. For a given half-sarcomere, if the number of interacting motors increases relative to the previous time step, additional motors, each in State 0, are incorporated into the model. If the number of interacting motors decreases relative to the previous time step, then the appropriate number of motors are removed.

(iii) **Allow the state of each motor to evolve.**

Each interacting motor within a half-sarcomere starts in State $l$, where $l = 0, 1, or 2$. The probability of moving to the next state in the forward direction is given by

$$p_{\text{forward}} = k_l \Delta t,$$

and the probability of moving to the previous state in the backward direction is given by

$$p_{\text{backward}} = k_{-l} \Delta t.$$  

The probability of remaining in the same state is thus given by

$$p_{\text{remain}} = 1 - p_{\text{backward}} - p_{\text{forward}}.$$  

For each motor, a number $n_{\text{rand}}$ is selected at random between zero and one, and a decision is made based on the following rules:

- The motor transitions from State $l$ to State $(l + 1)$ if $n_{\text{rand}} < p_{\text{forward}}$
- The motor transitions from State $l$ to State $(l - 1)$ if $p_{\text{forward}} < n_{\text{rand}} < p_{\text{forward}} + p_{\text{backward}}$
- The motor remains in State $l$ if $n_{\text{rand}} > p_{\text{forward}} + p_{\text{backward}}$

$$
\begin{align*}
\text{Within half-sarcomere } i, \text{ we also define a term } \varepsilon_{n,i}, \text{ which describes the extension of the } n\text{th elastic cross-bridge linkage away from its equilibrium position. For a motor in State 0, } \varepsilon_{n,i} \text{ is set to zero. For a motor that transitions from State 1 to 2, } \varepsilon_{n,i} \text{ increases by } d \text{ (indicating a power stroke). For a } \varepsilon_{n,i} \text{ increases by } d \text{ (indicating a power stroke). For a }}
\end{align*}
$$
A motor that has just entered State 1 or 2 from State 0 is transitioning from a weakly actin-bound state to a strongly bound state. Due to thermal motion, the motor may not bind at zero extension. To account for this, we assign a value for $\varepsilon_{n,i}$ that is selected from a Gaussian distribution with mean zero and standard deviation of 7.5 nm.

(iv) **Allow the myofibril to return to mechanical equilibrium.**

As a result of the motors’ changing states, the system may be out of mechanical equilibrium. We allow the boundaries of the half-sarcomeres ($y_j$) to move to return to equilibrium. The only exception is that we do not allow $y_0$ or $y_N$ (the ends of the myofibril) to move.

Specifically, before the boundaries are allowed to move, boundary $y_j$ experiences a force in the negative direction from the half-sarcomere to its left. This force has magnitude

$$F_{\text{left},j} = F_{\text{nonlinear}}(x_{hs,j-1}) + \sum_n \kappa_{cb} \varepsilon_{n,j-1},$$

(22)

where the summation is over all the interacting cross bridges. It also experiences a force in the positive direction due to the half-sarcomere to its right, which has magnitude

$$F_{\text{right},j} = F_{\text{nonlinear}}(x_{hs,j}) + \sum_n \kappa_{cb} \varepsilon_{n,j},$$

(23)

As already discussed, as a result of the evolution of the motors in step (iii), these two forces may not be in balance. We thus allow all the boundaries to move until mechanical equilibrium is restored, and $F_{\text{left},j}$ is equal to $F_{\text{right},j}$ for each boundary. To do this, we define the required displacement of the boundary $y_j$ as $\Delta y_j$. The length of each half-sarcomere $x_{hs,i}$ changes as a result of these displacements according to the relation

$$\Delta x_{hs,i} = \Delta y_{i+1} - \Delta y_i$$

(24)
When the half-sarcomere changes its length, this also changes the extension of all attached motors (in States 1 and 2) by $\Delta x_{hs,i}$. For half-sarcomere $i$, we define the number of these attached motors as $N_{attached,i}$.

As a result of these changes in the half-sarcomere lengths, the forces in equations 22 and 23 become

\begin{equation}
F_{left,j} = F_{nonlinear}(x_{hs,j-1} + \Delta x_{hs,j-1}) + \sum_n \kappa_{cb} \varepsilon_{n,j-1} + N_{attached,j-1} \kappa_{cb} \Delta x_{hs,j-1} \tag{25}
\end{equation}

\begin{equation}
F_{right,j} = F_{nonlinear}(x_{hs,j} + \Delta x_{hs,j}) + \sum_n \kappa_{cb} \varepsilon_{n,j} + N_{attached,j} \kappa_{cb} \Delta x_{hs,j} \tag{26}
\end{equation}

For each boundary, we require $F_{left,j} - F_{right,j} = 0$, which results in $N - 1$ equations which all must be satisfied at mechanical equilibrium (note that we do not have an equation for either the $y_0$ or $y_N$ boundaries). We use a non-linear solver in Python to find the values of $\Delta y_j$ which satisfy these equations (59).

Once this process is complete, we modify our boundary positions using the resulting displacements, and we return to step (ii). This process is repeated for five seconds.

**B) Isometric activation of a myofibril at an average half-sarcomere of length 1.4 $\mu$m, followed by a stretch of 0.1 um/half-sarcomere**

The initial conditions and the initial isometric activation occur identically as described above in (i), except that $x_{hs,i} = 1.4 \mu m$. At one second, a stretch of the myofibril occurs at a rate of 0.1 $\mu$m/s/half-sarcomere until the half-sarcomeres reach an average length of 1.5 $\mu m$ (after 1 second). To achieve this, during the stretch, each time-step starts with a displacement of the boundary at the end of the myofibril ($y_N$). For a myofibril that consists of $N$ half-sarcomeres, this extension is given by $0.1N\Delta t \mu m$. We then allow the system to return to mechanical equilibrium, as described earlier. Following this, we proceed through steps (ii-iv) from above.

Once the stretch is complete, we return to the isometric protocol described in (A), and continue until a total time of five seconds had elapsed. Because we assume that heterogeneities in half-sarcomere lengths arise during stretch, $\propto = 0$ during isometric conditions, and $\propto = 0.2$ during the stretch.

**C) Stretch of a myofibril, where the stiffness of the non-linear element increases**
The same protocol as (B) is followed, except that, upon initiation of the stretch, the value of $\sigma$ in equation 12 is increased by a factor of 1.5.

RESULTS

**Intact fibers.** When the intact fibers were activated at $L_o$, they produced forces of approximately 140 mN/mm² (Figures 1 and 3), a value that is consistent with previous studies using the diaphragm from mice (4). The fibers underwent several contractions without a loss of contractile capacity. In all bundles investigated in this study, the steady-state forces following active stretch were greater than the isometric force during reference contractions at the corresponding length ($L_f$) (Fig. 3).

This result was observed for both stretch amplitudes used in this study (4% $L_o$ and 8% $L_o$). In Fig. 3, it is clear that this RFE is larger when the 8% stretch was applied to the bundles, a result that has been observed previously, i.e. the RFE is dependent on the magnitude of stretch. Note that when the contractions are developed at a longer length, there is a large passive force developed by the fibers, but the RFE is still present (Fig. 3B). In all experiments, the steady-state forces following active stretch were always equal or greater than the isometric reference contractions at the length from which the stretch was initiated ($L_i$) (Fig. 3C).

In order to represent the relation between force produced by the fibers and the length, we plotted all the experiments after binning the data into sets related to forces produced at the initial length ($L_i$) and at $L_o$ (Fig. 4A -B).

It is clear that a 4% $L_o$ stretch induced a lower RFE than that obtained during an 8% $L_o$ stretch, but while the former tended to remain relatively constant for all the lengths progressively tested; the latter rapidly reached its highest value around the 85% of $L_o/P_o$, and presented a tendency to decrease quickly, paralleling the 4% values to basically similar values at the last $L_o/P_o$ value investigated (Fig. 4). It is important to note that when the fibers were activated at longer lengths, the
force decreased to levels that follow closely the force-length relation for striated muscles (40), and consistent with other studies that have evaluated this relation for the diaphragm.

Figure 4C summarizes the levels of RFE observed in all conditions investigated in this study; at different L_i/Po and with the two amplitudes of stretch (4% and 8%). The RFE was larger with 8% stretches across all lengths, with a significant difference observed at around 85% of L_i/Po.

**Myofibrils.** The force produced by myofibrils maximally activated with a pCa^{2+} 4.5, at a sarcomere length 2.7 μm was 136 ± 24 nN/μm^2, in the same range as previous studies with myofibrils isolated from the diaphragm (4) or other skeletal muscles (11, 21). When the myofibrils were activated at longer sarcomere lengths (2.8 μm, 2.9 μm, 3.0 μm and 3.1μm), the force decreased to levels corresponds to those predicted by the force-sarcomere length relation (13, 40) (Figs. 5, 6 and 7).

Figure 5 shows force traces recorded during a typical experiment with myofibrils in which they were either activated isometrically or stretched during activation. In this case, myofibrils were stretched from 2.8 μm to 2.9 μm, or from 2.8 μm to 3.0 μm. It is clear that when myofibrils were stretched during activation, RFE was present (Fig. 5A-B), a result that was observed consistently across the experiments, and similar to the results observed with intact fibers. The RFE was larger with larger stretches, in agreement with the results obtained with the intact fibers (Figs. 6A-B and 7A-B).

Since the force traces are not completely parallel after the stretch, and we cannot activate fibers and myofibrils for very long periods of time without causing damage or fatigue to the preparations, we have performed the following analysis to evaluate when the RFE would cease. We plotted the difference between the stretch forces and the isometric forces from the time the stretch stopped to the time we deactivated the preparations (Fig. 5). We then fitted a third-order polynomial equation to the curve and interpolated to the X-axis where force reached zero. In the case of Fig. 5C, the force difference drops to zero at 15.3 seconds after the end of the stretch. Figure 6 shows the average values obtained with these plots for different conditions investigated in this study, and shows that force remains elevated for a long period of time.
Sarcomere length dispersion. We measured the dispersion of sarcomere lengths in all myofibrils investigated in this study (Figs. 6C-D and 7C-D). The sarcomere length dispersion provides an indication of the non-uniformity of sarcomere lengths induced by activation and stretch of the sarcomeres. The dispersion was small in rested myofibrils, as expected from previous studies. There was an increase in the SL dispersion during activation, i.e. during maximal force development in isometric conditions. The increase is consistent with previous studies performed with skeletal muscle myofibrils (13, 20, 21, 42). Most importantly, there was a significant increase in the level of sarcomere length non-uniformity after the stretches were applied to the myofibrils. Similar to what we observed with the force developed by the myofibrils, the increase in sarcomere length dispersion was larger for the stretches performed with larger magnitudes (i.e., equivalent to stretches performed with 8% L₀ compared to 4% L₀).

Force enhancement and Ca²⁺ sensitivity. We also tested the myofibrils at 2.9 μm but at different Ca²⁺ concentrations (pCa²⁺). Fig. 9 shows that the force-pCa²⁺ relation is shifted to the left when stretch contractions are induced to the myofibrils, indicating an increase in the Ca²⁺ sensitivity of the contractile apparatus. The shift was tested statistically by comparisons of the pCa²⁺₅₀ in the two curves, i.e., the amount of Ca²⁺ required to produce 50% of maximal force. The shift was significant with pCa²⁺₅₀ values of 6.08 ± 0.53 and 5.86 ± 0.25 for the RFE and the isometric contractions, respectively.

Stochastic model. In Fig. 10, we present the results of our 5 second simulations for a myofibril consisting of 50 half-sarcomeres. When we compare the force produced by a myofibril that is activated isometrically at an average half-sarcomere length of 1.5 μm (Fig. 10 top, black line) with a myofibril that is activated isometrically at 1.4 μm and then stretched to 1.5 μm (Fig.10 top, red line), we see a significant, persistent RFE. This is associated with an increase in the standard deviation of the half-sarcomere lengths for the stretched fiber (Fig. 9 bottom, red line). This effect is enhanced when we increase the stiffness of the non-linear passive spring upon initiation of the stretch (Fig. 10, blue line).
Taken together, these simulations support the hypothesis that RFE results from both (1) nonuniformity in half-sarcomere lengths resulting from stretch and (2) the increase in stiffness of titin that arises when it is stretched following activation.

DISCUSSION

In this study, we investigated the RFE in diaphragm muscle fibers and myofibrils, and its connection to sarcomere-length non-uniformities. We used two complementary techniques that allow the investigation of the RFE in intact fibers and permeabilized myofibrils. Both preparations showed that RFE was present in the diaphragm, and was dependent on the amplitude of the stretch applied to the preparation, such that large stretches led to higher increases in force. RFE showed a different force-length relation when stretches of different amplitudes were applied to the intact fibers. Furthermore, we observed that RFE was accompanied by (a) an increase in the degree of sarcomere length non-uniformity, and (b) an increase in the Ca\(^{2+}\) sensitivity of the contractile apparatus. We also developed a stochastic, mathematical model and showed that sarcomere non-uniformities induced during a stretch may indeed lead to a residual increase in force, and that can be augmented when we increase the stiffness of titin as a result of myofibril activation and stretch. These results suggest that the RFE is caused by sarcomere length non-uniformities and accompanied by a greater response of the myofibrils to activation, likely due to changes in the properties of titin.

In our experiments, the force developed during an isometric contraction was first allowed to stabilize, upon which stretches of % relative to the starting length of the fibres and myofibril were imposed. The forces obtained after stretch were between 3 and 24% greater than the isometric force developed at the same sarcomere length, similar to previous results (42, 48, 50). Although there are not many studies that carefully compared forces produced by skeletal muscles during contractions while controlling for sarcomere length and sample integrity, those that use a rigorous experimental protocol report an average RFE of 5% - 40% in single fibers (11, 16, 22), myofibrils (42, 48, 50), sarcomeres (7) and half-sarcomeres (35, 48). Our results are particularly revealing because the results from mechanical measures done in intact fibers from the mice, activated through action potentials, were similar to those obtained with individual myofibrils, activated directly with Ca\(^{2+}\).
Interestingly, studies with myofibrils and intact muscles isolated from the heart do not show RFE. They show that stretch increased the force but just after the stretch the force decays to attain a similar level to that observed during regular contractions. The difference in the RFE between skeletal muscles and cardiac muscles has been explained by one of the underlying mechanisms of RFE: an increase in the stiffness of titin upon activation and stretch. The isoforms of titin in skeletal (limb and diaphragm) and cardiac muscles are different. We suggest that activation affects N2A titin isoforms of skeletal myofibrils causing RFE after stretch, but not N2B titin isoforms of cardiac myofibrils, as follows. When the myofibrils are activated, the released Ca$^{2+}$ binds to the I-band region of titin containing proline, glutamate, valine and lysine (PEVK) which are rich with glutamate domains (E-domains) (27). Consequently, titin reduces its persistence length (25, 53-55), increases its stiffness (27), and produces more force when stretched. The N2A titin isoforms of the skeletal psoas myofibrils have larger PEVK segments with more E-domains than the N2B titin isoforms of the cardiac papillary myofibrils (5, 28). Therefore, skeletal psoas myofibrils are more likely to show an increase of titin force during activation than cardiac myofibrils.

This mechanism aligns with recent studies that show a static tension and directly associate it with residual force enhancement in skeletal muscles (11, 37, 46), but not in cardiac muscles (11, 50). The static tension represents an increase in forces that are not derived from cross-bridges, but a passive component, likely due to titin – that is evident when skeletal muscles are activated and stretched in the absence of myosin-actin interactions (38). RFE has been a subject of much investigation because it cannot readily be explained within the features of the classic isometric force-sarcomere length relation (3, 18).

There is also the possibility that Ca$^{2+}$ increases the binding between actin and titin, which would increase the sarcomere stiffness and consequently cause the RFE. It is known that titin binds to actin in PEVK segments and that this binding is regulated by Ca$^{2+}$ (23, 24, 26, 31, 61). However, there are studies that show an increase in actin motility over myosin in the presence of titin and Ca$^{2+}$ which suggests that the binding between titin and actin is weakened (52) (26). Another study showed that S100A1, a soluble Ca$^{2+}$-binding protein highly concentrated in striated muscles hinders actin from interacting with PEVK segments of titin (61), which would prevent an increase of stiffness. Ultimately, the effect of Ca$^{2+}$ on titin binding to actin seems to be inconclusive and requires further investigation.
Intact fibers and different stretch magnitudes. During all consecutive stretches applied to the intact fibers, RFE was present along the entire force-length relation (data not shown). Since this study focused on the RFE developed on the descending limb of the force-length relation, only RFE values starting from $L_i$ corresponding to $\leq 95\% P_o$ were collected for analysis. For all $L_i/P_o$ ratios examined and for both stretch amplitudes tested, RFE was always present but it showed two distinct force-length curves, especially in the first section, after which RFE showed a common trend, to become negligible.

The force-length curves (with 4% or 8% stretches) were different particularly at shorter lengths. The RFE after stretches of 4% $L_o$ remained constant for all the $L_i/P_o$ tested, showing that this kind of preparation could well tolerate a certain amount of stretch without losing its capacity to generate active force. In experiments in which we used stretches of 8% $L_o$, the RFE reached a maximum level in the first part of the descending limb of the force-length relation, and then it decreases progressively. These results were expected, and consistent with previous findings showing that there is an optimal length for the development of RFE, which is around $2.8\mu m – 3.0\mu m$ or just beyond the plateau of the force length relation (16, 48). In one of these studies, Edman et al. (16) used intact fibers from the frog, a preparations similar to the one we used in this study, and showed results similar to ours – a dependence of the RFE on the stretch magnitude and the initial sarcomere length (their figure 5).

Sarcomere length non-uniformity. The mechanisms of RFE have been clarified over the past years, and now it is clear that the increase in force is caused partially by sarcomere length non-uniformities that arise upon contraction and stretch of a myofibril (16, 21, 22, 35). When activated, sarcomeres at different lengths elicit different responses – shorter, stronger sarcomeres will contract more quickly, at the expense of longer, weaker sarcomeres that will stretch (17, 22). Imposing a stretch during an active contraction exaggerates these irregularities in sarcomere lengths; strong sarcomeres will have a favourable overlap, producing higher active forces, while weak sarcomeres elongate to greater lengths shifting their contribution to the overall force output increasingly towards large passive forces. This scenario allows for an increase in the total force: shorter sarcomeres have a larger number of myosin heads attached to actin, producing more active force. The sarcomeres that will be stretched lose filament overlap but will have a greater stiffness due to the elongation of titin, which acts as a spring-like molecule and is responsible for passive forces in sarcomeres. The overall force produced by both the shorter sarcomeres with more cross-bridge
interaction and the longer sarcomeres with a large passive force will be higher than the force produced during isometric contractions.

We observed that the RFE was accompanied by an increase in sarcomere length non-uniformity, a result that has been observed previously in myofibrils from the psoas muscle (21). In that study, we induced non-uniformity in myofibrils by depleting the thick filament from a target sarcomere, which increased significantly the sarcomere length non-uniformity. The result was a decline in isometric force with the inactivation of contractile proteins in one sarcomere, and an increase in non-uniformity. Most importantly, the RFE was ~20% higher in these myofibrils compared to the isometric force produced at the same sarcomere length. While control myofibrils produce a residual force enhancement of ~5%, myofibrils with one inactive sarcomere show four times larger levels of residual force enhancement (21).

Some myofibrils in the current study showed a negligible amount of sarcomere length non-uniformity, and yet still produced a small degree of RFE. These results suggest that non-uniformity is, in part, connected to residual force enhancement, but that it cannot account for the phenomenon in its entirety. Furthermore, the occurrence of RFE in single sarcomeres and half-sarcomeres - preparations in which non-uniformity is naturally absent - also suggests an alternate mechanism for enhanced forces after stretch (35, 48).

A possible explanation is centered around the Ca\(^{2+}\)-dependent increase in stiffness of the elastic protein titin. When Ca\(^{2+}\) levels rise during activation of myofibrils, Ca\(^{2+}\) binds to the PEVK-domains of titin, which consequently reduces its persistence length (27). This leads to an increase in the stiffness of titin and a subsequently higher contribution of passive forces to the total force output after an imposed stretch. When a myofibril is stretched, the stiffness of titin will increase further causing a larger level of passive force contribution to the total force. This mechanism has been investigated in recent studies that linked levels of a static tension, that cannot be accounted for myosin-actin interactions, to the amount of residual force enhancement seen in myofibrils and muscle fibers (11, 12, 38). Interestingly, myofibrils isolated from muscles containing different titin isoforms present different responses to stretch. Studies specifically designed to compare different muscle types showed that myofibrils isolated from the soleus and psoas show residual force enhancement that is accompanied by an increase in the static stiffness, while cardiac myofibrils do not show residual force enhancement (11, 50). The difference may be related to the physiological role. Cardiac muscles are not stretched while contracting in vivo, and thus a residual force
enhancement caused by a Ca²⁺-dependent increase in titin stiffness would not play a physiological role during contractions. This interpretation is strengthened by a recent study showing that intact cardiac trabeculae also do not present a residual force enhancement after stretch (57).

Tellingly, our stochastic model showed that force enhancement can be produced by myofibrils when (i) we induce sarcomere length non-uniformities, and (ii) when we increase the static stiffness, or the stiffness produced by titin during activation and stretch. In the latter case, we observed larger levels of RFE, strengthening our hypotheses.

Ca²⁺ sensitivity of the contractile apparatus. The Ca²⁺ sensitivity was increased in myofibrils during the RFE, showing that the stretch leads to a greater force produced for a given level of Ca²⁺ activation. The increased Ca²⁺ sensitivity is on line with two mechanisms already discussed in this paper: an increase in sarcomere length non-uniformity and an increase in the titin-based increase in stiffness – which have been recognized as the two mechanisms of RFE (43, 45). First, there is a length-dependence of the force-pCa²⁺ relationship (32-34), such that sub-maximal contractions increase their force when activated at long sarcomere lengths, even with a decrease in filament overlap. Thus, an increase in individual sarcomere lengths caused by sarcomere length non-uniformity, would increase the Ca²⁺ sensitivity of the myofibrils. Second, the titin-induced passive force is an important contributor to the increase in Ca²⁺ sensitivity at long sarcomere lengths (8, 56, 58). Although there are no other studies investigating the Ca²⁺ sensitivity of the contractile apparatus in myofibrils, it has been shown that the level of muscle activation influences directly the RFE; stretches invoked in low Ca²⁺ concentrations lead to an larger RFE than large Ca²⁺ concentrations (36).
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Figure legends.

Figure 1: Typical contractions produced by a bundle of intact fibers, developed at $L_f$ and at $L_i$ + 4%, starting from the same $L_i$. The solid line indicates the isometric contraction, and the dotted line indicates the contraction with an active stretch imposed during activation. Stretch amplitude was 4%. A and B indicate the points where the isometric forces were measured, at 100 ms before the end of stimulation.

Figure 2: (A) Chemomechanical cycle for an individual cross-bridge in an activated sarcomere. The transition from State 1 to State 2 is associated with the motor's power stroke. (B) Mechanical model for a single activated half-sarcomere, consisting of numerous cross-bridges (represented by red springs) connected in parallel with one another. The cross-bridges are also connected in parallel with a single passive element (titin; represented by a black spring). (C) Model of a myofibril has a series of $N$ half-sarcomeres in series. The positions of the boundaries are represented by $y_j$, where $j = 0, 1, 2 \ldots N$.

Figure 3: Panel (A) and (B) shown typical force traces from the same fiber bundle during passive and active stretches at 4% and 8% $L_0$ stretch amplitude, with stretch durations of 0.2 and 0.4 sec, respectively. In panel (C), contractions reflect the isometric force produced at optimum length ($P_o$, top isometric trace), the isometric force at the starting length ($L_i$, middle isometric trace) and the isometric force produced at the final length ($L_f-p$, bottom isometric trace). It also shows the active stretch ($L_i$ + 4%, dotted line) and the passive stretch ($p$, bottom trace, traced line).

Figure 4: Panels A and B show the mean values ($\pm$ S.E.M.) for all experiments conducted with the intact fibers, during isometric (squares) and stretch (dots) contractions, in all $L_i/P_o$ ratios investigated. Stretches were performed with an amplitude of 4% (A) or 8% (B) of $L_0$. The black triangle indicates the tension at $P_0$. In panel C, the RFE average values of each group are shown. RFE values were obtained subtracting $L_i$ + % from the respective $L_f$ averaged values. Dots and squares represent the values obtained with stretches of 4% and 8% of $L_0$ respectively, for different $L_i/P_o$ ratios. The p-values obtained during comparisons performed after ANOVA are show for each condition.

Figure 5: Typical myofibril contractions recorded during an experiment in which they were activated isometrically (solid lines) or stretched during activation (dotted lines). The traces show isometric forces developed at 2.9 $\mu$m, and forces developed when myofibrils were stretched from 2.8 $\mu$m to 2.9 $\mu$m (A), and isometric forces developed at 3.0 $\mu$m and when myofibrils were stretched from 2.8 $\mu$m to 3.0 $\mu$m (B). It is clear that myofibrils developed RFE in both conditions.
Figure 6: Mean force values of myofibrils produced during isometric contractions developed at 2.8 μm and 2.9 μm (solid lines, A and B) and after stretch contractions initiated at 2.7 μm (dotted lines, A and B). The force is significantly higher after stretch in both conditions. The level of sarcomere length non-uniformity during these contractions is represented and (C) and (D). There is a significant increase in the degree of sarcomere length non-uniformity in myofibrils when they are at rest, during activation and then after the stretch the results in RFE. The p-values obtained during comparisons performed after ANOVA are shown for each condition.

Figure 7: Mean force values of myofibrils produced during isometric contractions developed at 2.9 μm and 3.0 μm (solid lines, A and B) and after stretch contractions initiated at 2.8 μm (dotted lines, A and B). The force is significantly higher after stretch in both conditions. The level of sarcomere length non-uniformity during these contractions is represented and (C) and (D). There is a significant increase in the degree of sarcomere length non-uniformity in myofibrils when they are at rest, during activation and then after the stretch the results in RFE. The p-values obtained during comparisons performed after ANOVA are shown for each condition.

Figure 8: Time in which the RFE dissipates after the stretch in the different conditions investigated in this study, in experiments with fiber bundles (A) and myofibrils (B). The results were obtained after interpolating the differences between the stretch curves and the isometric curves until force reached zero. They represent the average of all curves plotted for a given situation. The X-axes show the different situations investigated during the experiments.

Figure 9: The force-pCa²⁺ relation derived from force values developed during isometric contractions (solid line) and after stretches (dotted line). All values were recorded at a final sarcomere length of 2.9 μm. There is shift in the curve to the left, indicating that the Ca²⁺ sensitivity of the contractile apparatus is increased in conditions of RFE. The pCa²⁺₅₀ was significantly different between the two conditions (p = 0.01).

Figure 10. Time traces of the stress (top) and standard deviation of half-sarcomere length (bottom) for a myofibril consisting of 50 half-sarcomeres. The black curve is for a myofibril that is activated under isometric conditions, with an average half-sarcomere length of 1.5 μm. The red curve describes a myofibril that is first activated under isometric conditions, with an average half-sarcomere length of 1.4 μm, and then stretched starting at t = 1 second at a rate of 0.1 μm/s/half-sarcomere until it reaches an average half-sarcomere length of 1.5 μm. The blue curve describes a myofibril that undergoes the same stretching process as described by the red curve, except that the stiffness of the non-linear passive spring is increased upon initiation of the stretch.


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A

B

C
A

Time (sec) for RFE = 0

- 4% stretch
- 8% stretch

B

Time (sec) for RFE = 0

Stretches:
- 2.7-2.8um
- 2.7-2.9um
- 2.8-2.9um
- 2.8-3.0um