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RESEARCH ARTICLE

Effect of active shortening and stretching on the rate of force re-development in rabbit psoas muscle fibres

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ABSTRACT

The steady-state isometric force produced by skeletal muscle after active shortening and stretching is depressed and enhanced, respectively, compared with purely isometric force produced at corresponding final lengths and at the same level of activation. One hypothesis proposed to account for these force depression (FD) and force enhancement (FE) properties is a change in cross-bridge cycling kinetics. The rate of cross-bridge attachment (*f*) and/or cross-bridge detachment (*g*) may be altered following active shortening and active stretching, leading to FD and FE, respectively. Experiments elucidating cross-bridge kinetics in actively shortened and stretched muscle preparations and their corresponding purely isometric contractions have yet to be performed. The aim of this study was to investigate cross-bridge cycling kinetics of muscle fibres at steady-state following active shortening and stretching. This was done by determining muscle fibre stiffness and rate of active force redevelopment following a quick release–re-stretch protocol (k_{TR}). Applying these measures to equations previously used in the literature for a two-state cross-bridge cycling model (attached/detached cross-bridges) allowed us to determine apparent *f* and *g*, the proportion of attached cross-bridges, and the force produced per cross-bridge. k_{TR}, apparent *f* and *g*, the proportion of attached cross-bridges and the force produced per cross-bridge were significantly decreased following active shortening compared with corresponding purely isometric contractions, indicating a change in cross-bridge cycling kinetics. Additionally, we showed no change in cross-bridge cycling kinetics following active stretch compared with corresponding purely isometric contractions. These findings suggest that FD is associated with changes in cross-bridge kinetics, whereas FE is not.

KEY WORDS: Force depression, Force enhancement, Cross-bridge cycling kinetics, Cross-bridge theory, Proportion of attached cross-bridges, Force per cross-bridge

INTRODUCTION

It has been shown consistently that the steady-state isometric force produced after active muscle shortening or stretching is smaller or greater, respectively, than the steady-state force produced by purely isometric contractions at the same final length and level of activation (for review, see Herzog, 2017). The decrease in steady-state isometric force following active shortening and the increase in steady-state isometric force following active stretching have been termed force depression (FD) and force enhancement (FE), respectively. FD and FE are universal properties of striated muscles that have been observed in human, rabbit, cat and amphibian skeletal muscles (Abbott and Aubert, 1952; De Ruiter et al., 1998; Herzog and Leonard, 2002; Maréchal and Plaghki, 1979; Mashouri et al., 2021; Morgan et al., 2000; Seiberl et al., 2015; Sugi and Tsuchiya, 1988), and rabbit cardiac muscle (Boldt et al., 2020). FD and FE occur in sub-maximal voluntary contractions within human adductor pollicis (Oskouei and Herzog, 2006; Rousanoglou et al., 2007), indicating that these properties may have a role in everyday movements and coordination. Interestingly, Power et al. (2012) showed that eccentric strength was maintained in older adults despite a significant loss in voluntary maximal isometric and concentric contractions, indicating that active stretch and FE likely contribute to the maintenance of force in eccentric movements in the elderly. Moreover, older adults show greater force reduction following active shortening compared with their young counterparts (Power et al., 2014), suggesting that FD contributes to the loss of force in concentric contractions in older adults. When testing muscle efficiency following active stretching and shortening, Joumaa et al. (2017) showed that while FD did not affect muscle efficiency, the increase in force observed in FE was accompanied by a decrease in ATP consumption per unit of force, suggesting that FE increases muscle efficiency (Joumaa and Herzog, 2013). These findings suggest that FD and FE could regulate muscle force production and energy consumption, and therefore should be considered when analysing everyday movements. Furthermore, FD and FE research may provide insight into mechanisms responsible for force loss in aging and various muscle disease conditions, which may eventually lead to the development of measures preventing sarcopenia and other muscle degenerative processes.

Our current understanding of muscle contraction is based on the cross-bridge theory (Huxley, 1957). According to this theory, muscle contraction and active force production occur through the sliding of thin and thick filaments and actin–myosin cross-bridge cycling. FD and FE are perplexing properties of muscle contraction, as the cross-bridge theory requires that the steady-state active isometric force is proportional to the muscle final length and the degree of overlap between thin and thick filaments, independently of how the muscle reaches its final length (Gordon et al., 1966; Huxley, 1957).

Several hypotheses have been developed to account for FD and FE. The sarcomere length (SL) non-uniformity hypothesis has received the greatest acceptance. This hypothesis suggests that because sarcomeres are unstable on the descending limb of the force–length relationship (Hill, 1953), they shorten and stretch by different amounts during dynamic contractions on the descending limb of the force–length relationship (Julian and Morgan, 1979). This non-uniform sarcomere behaviour results in a situation where, at steady-state, actively shortened and stretched contractions produce

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force that is smaller or greater, respectively, than the force produced by isometric contractions at the corresponding fibre length. However, there has been little direct experimental evidence supporting this hypothesis. Active shortening and stretching experiments performed in single myofibrils, in which force and individual SLs were measured (Johnston et al., 2019; Joumaa and Herzog, 2013; Joumaa et al., 2008a; Telley et al., 2006) have shown that FD and FE occur in the absence of increased SL non-uniformities. The SL non-uniformity hypothesis is further challenged by results demonstrating that FE occurs in single, mechanically isolated sarcomeres (Herzog et al., 2010). These results suggest that FD and FE originate from structures within the sarcomeres.

Maréchal and Plaghki (1979) proposed an intra-sarcomeric mechanism for FD based on the idea of a stress-induced inhibition of cross-bridge attachment following active shortening. They suggested that actin filaments become strained during the initial activation and active shortening of the muscle fibre, and thus undergo conformational changes that inhibit normal cross-bridge attachment (Maréchal and Plaghki, 1979). Passive force produced from the engagement of an intra-sarcomeric passive element in actively stretched muscle fibres has been suggested to explain FE (Edman et al., 1978; Herzog and Leonard, 2002; Hessel et al., 2017; Noble, 1992; Pinniger et al., 2006). Another potential intra-sarcomeric mechanism leading to FD and FE is a change in cross-bridge cycling kinetics following active shortening and stretching (Rassier and Herzog, 2004). It is possible that cross-bridge cycling kinetics, indicated by the equilibrium between the rate of cross-bridge attachment (f) and the rate of cross-bridge detachment (g) (Huxley, 1957), are altered at steady-state after active shortening and stretching compared with the corresponding purely isometric contractions. As the rate of cross-bridge cycling heavily impacts the proportion of attached cross-bridges and the force produced per cross-bridge, changes in f and g could lead to FD and FE.

This hypothesis is supported by findings that FD is accompanied by a decrease in stiffness (Joumaa et al., 2021; Lee and Herzog, 2003; Sugi and Tsuchiya, 1988). Single muscle fibre stiffness has been thought to reflect the proportion of attached cross-bridges; therefore, changes in stiffness are assumed to be primarily caused by a change in the proportion of attached cross-bridges (Ford et al., 1981; Huxley and Simmons, 1971). This then suggests that actively shortened muscle has a decrease in the proportion of attached cross-bridges (compared with the corresponding purely isometric contraction), that is likely caused by changes in cross-bridge kinetics. This is further supported by findings from Corr and Herzog (2005) who showed that FD in cat soleus muscle is accompanied by a decrease in the rate of force recovery following shortening. Although actively stretched muscle is not accompanied by a change in stiffness (Joumaa et al., 2021; Sugi and Tsuchiya, 1988), a cross-bridge kinetic mechanism resulting in an increase in the force produced per cross-bridge has been suspected to contribute to FE (Koppes et al., 2013).

The rate of force redevelopment (k_{TR}), along with stiffness, has been used to determine f and g (Brenner, 1988). k_{TR} can be obtained after complete dissociation of cross-bridges between thick and thin filaments achieved through a quick release–re-stretch protocol (Brenner, 1988; Kreutziger et al., 2008). The redevelopment of force after this protocol allows for cross-bridge reattachment, and thus cross-bridge cycling kinetics, to be assessed independently of Ca^{2+} and troponin-tropomyosin regulation of actin–myosin interactions (Brenner, 1988; Kreutziger et al., 2008).

Although changes in cross-bridge kinetics have been suggested to explain FD and FE, experiments elucidating cross-bridge kinetics in actively shortened and stretched muscle preparations and their corresponding purely isometric contractions have not been performed. Therefore, the aim of this study was to investigate cross-bridge cycling kinetics in muscle fibres at steady-state isometric force following active shortening and stretching by determining k_{TR} and stiffness. Knowing that FD is associated with a decrease in the proportion of attached cross-bridges, while FE occurs without changes in the proportion of attached cross-bridges (Joumaa et al., 2021; Sugi and Tsuchiya, 1988), we hypothesized that: (i) active shortening is accompanied by a decrease in k_{TR} and stiffness, indicating a change in cross-bridge cycling kinetics compared with the corresponding purely isometric reference contraction, and that (ii) active stretching does not lead to changes in k_{TR}, stiffness and cross-bridge cycling kinetics compared with the corresponding purely isometric reference contraction.

MATERIALS AND METHODS

Muscle fibre preparation

Six-month-old New Zealand white female rabbits were euthanized according to a protocol approved by the University of Calgary's Life and Environmental Sciences Animal Care and Ethics Committee. Strips of psoas muscle were dissected, tied to wooden sticks, and chemically skinned using standard protocols (Mounier et al., 1989). Briefly, samples were stored in a relaxing solution (see 'Solutions' below) for 12 h at 4°C, and then transferred to a relaxing:glycerol (50:50) solution for 2 weeks at −20°C before use in experiments (Leonard and Herzog, 2010). When performing experiments, a single muscle fibre was isolated from the skinned psoas muscle strip and transferred to an experimental glass chamber containing relaxing solution. The ends of the fibre were then glued (utilizing cellulose acetate sheets and acetone), one end to the hook of a length controller (Aurora Scientific, Model 322C, Aurora, ON, Canada) and the other to the hook of a force transducer (Aurora Scientific, Model 402A, Aurora, ON, Canada). This allowed for control of the fibre length and measurement of the fibre force. Fibres (n=12) were set at an average SL of 2.4 µm using a He-Ne laser (Edman and Flitney, 1982). Active shortening and lengthening experiments were then performed on all fibres in the order shown below. This order was chosen to reduce the impact of potential fatigue on our results and to underestimate FD and FE properties in case force decreased over time following multiple trials. All experiments were performed at room temperature (~22°C).

Active shortening experiments

Active shortening contraction from SL=3.0 µm to SL=2.4 µm

Skinned fibres were stretched passively from an average SL of 2.4 µm to an average SL of 3.0 µm over 5 s in a relaxing solution and held until steady-state was reached. Fibres were then activated using an activating solution (see below), actively shortened to an average SL of 2.4 µm in 5 s and held at this length until steady-state had been reached (Fig. 1A).

Reference contraction at SL=2.4 µm

After a 5 min rest period in a relaxing solution, the fibres were activated isometrically at an average SL of 2.4 µm (Fig. 1B).

Active stretch experiments

Reference contraction at SL=3.0 µm

After a 5 min rest period in relaxing solution, the fibres were stretched passively from an average SL of 2.4 µm to an average SL

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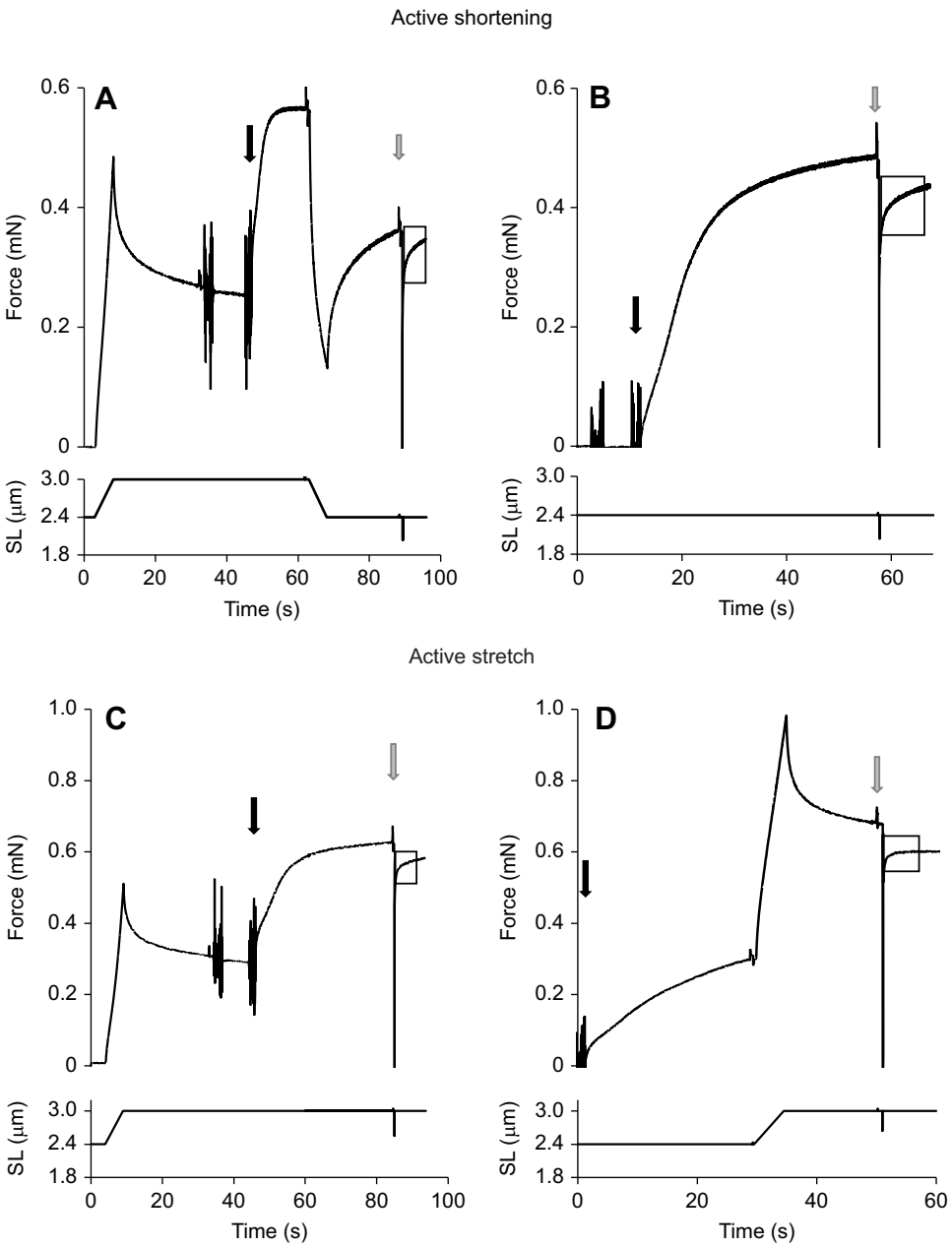

Fig. 1. Force as a function of time for reference, active shortening and active stretch contractions. (A) In the active shortening contraction, the fibre was passively stretched from an average sarcomere length (SL) of 2.4 to 3.0 μm, activated and then actively shortened to an average SL of 2.4 μm. (B) In the reference contraction for active shortening experiments, the fibre was activated at an average SL of 2.4 μm. (C) In the reference contraction for active stretch experiments, the fibre was passively stretched from an average SL of 2.4 μm to an average SL of 3.0 μm and then activated. (D) In the active stretch contraction, the fibre was activated at an average SL of 2.4 μm and then actively stretched to 3.0 μm. Activation occurred when the relaxing solution in the testing chamber was replaced by a washing solution (free of EGTA and calcium) and then an activating solution (pCa 4.2). The noise in the graphs indicates the time when the solutions were changed. The black and grey arrows indicate when the fibre was activated and when stiffness was measured, respectively. The black boxes at the end of the tests indicate the region where k_{TR} was determined.

of 3.0 μm in 5 s and held until steady-state was reached. The fibres were then activated (Fig. 1C).

Active stretch contraction from SL=2.4 μm to SL=3.0 μm

After a 5 min rest period in relaxing solution at an average SL of 2.4 μm, fibres were activated and actively stretched from an average SL of 2.4 μm to an average SL of 3.0 μm in 5 s and held at this length until steady-state had been reached (Fig. 1D).

Stiffness measurements

Fibre stiffness was determined using a quick stretch–release cycle of 0.2% of the initial fibre length (fibre length at SL=2.4 μm) using a quick length-step (~0.6 ms). The high speed and low magnitude of stretch allows for cross-bridges to be stretched while still attached (Ford et al., 1981). This approach guarantees a nearly constant stiffness throughout the stretch phase (Huxley and Simmons, 1971). Stiffness was measured once the isometric steady-state force had been reached after active shortening and stretching, and at steady-state force production during the purely isometric reference contractions (Fig. 1).

k_{TR} measurements

The rate of force redevelopment (k_{TR}) was determined by fitting the time course of force redevelopment in the 1 s following the quick release–re-stretch protocol with a mono-exponential function (please see 'Analysis' section) (Brenner, 1988; Kreutziger et al., 2008). Fibres were rapidly (length-step ~0.6 ms) released by 15% of the initial fibre length, held for 20 ms, re-stretched by 20% of the initial fibre length, and then released (length-step ~0.6 ms) by 5% of the initial fibre length. This release–re-stretch protocol, as opposed to a release step alone, has been shown to prevent variation in SLs induced by fibre shortening alone (Brenner and Eisenberg, 1986). Furthermore, the quick stretch step is important to forcibly detach all cross-bridges that may not have detached during the

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shortening phase, and therefore, allows cross-bridges to reattach during the force redevelopment phase (Brenner and Eisenberg, 1986). The quick release–re-stretch protocol was performed following the stiffness test in active shortening and stretching, and the purely isometric reference contractions (Fig. 1).

Solutions

Relaxing solution consisted of potassium propionate (170 mmol l^{-1}), magnesium acetate (2.5 mmol l^{-1}), MOPS (20 mmol l^{-1}), ethylene glycol bis (2-aminoethyl ether)–N,N,N′,N′-tetraacetic acid (EGTA; 5 mmol l^{-1}), adenosine triphosphate (ATP; 2.5 mmol l^{-1}) and protease inhibitors (Complete®, Roche Diagnostics, Montreal, QB, Canada; 1 tablet for 100 ml of solution) at a pH of 7.0.

Washing solution was potassium propionate (185 mmol l^{-1}), magnesium acetate (2.5 mmol l^{-1}), MOPS (20 mmol l^{-1}), ATP (2.5 mmol l^{-1}), at a pH of 7.0. Activating solution was potassium propionate (170 mmol l^{-1}), magnesium acetate (2.5 mmol l^{-1}), MOPS (10 mmol l^{-1}), ATP (2.5 mmol l^{-1}) and free Ca^{2+} (buffered with EGTA until the solution reached a pCa (–log[Ca^{2+}]) of 4.2) at a pH of 7.0.

Analysis

Force

Force and length data were collected at a frequency of 20 kHz (Aurora Scientific, Model 600A, Aurora, ON, Canada). FD and FE were determined utilizing a custom-written MATLAB software program (The MathWorks Inc., Natick, MA, USA). FD was determined as the difference in steady-state force at a SL of 2.4 µm, between the active shortening contraction and the purely isometric reference contraction. FE was determined as the difference in steady-state force at a SL of 3.0 µm, between the active stretch contraction and the purely isometric reference contraction. Steady-state force was determined by averaging the force produced during 1 s before the stiffness test.

Stiffness

Stiffness was determined as the difference between the peak force obtained during the quick stretch–release cycle and the steady-state force before stretch ($\Delta\mathbf{F}=\mathbf{F}_{peak}-\mathbf{F}_{before}$; Fig. 2) divided by the amount of stretch (ΔL).

k_{TR}

k_{TR} was determined using SigmaPlot (v. 14.0, 2021). The force produced during 1 s immediately after the quick release–re-stretch protocol was fitted with a mono-exponential curve (Fig. 3), which was in excellent agreement with experimental data in all cases (R^2>0.95). k_{TR} was solved from the mono-exponential regression curve using the following equation (where a is a constant and t is time) (Brenner and Eisenberg, 1986):

$$\mathbf{F} = a(1 - e^{(k_{TR} \times t)}). \quad (1)$$

f and g

We used equations available in the literature (Brenner, 1988; Huxley, 1957) and derived from a two-state cross-bridge cycling model to determine the apparent rate constant of cross-bridge attachment (f) and the apparent rate constant of cross-bridge detachment (g). This cross-bridge cycling model includes an attached force-generating state and a detached non-force-generating state. f and g are termed 'apparent' rate constants because it is well accepted that the entire cross-bridge cycle comprises multiple detached and multiple attached cross-bridge states that are all represented here in a single detached and single attached state (Brenner, 1988). Despite this simplification, cross-bridge cycling can be well represented by f and g. Assuming that the reverse transitions f^{-1} and g^{-1} are negligible (Brenner, 1988), the steady-state proportion of cross-bridges in the attached force-generating state (α) was determined using Eqn 2 (Brenner, 1988; Huxley, 1957):

$$\alpha = \frac{f}{f + g}, \quad (2)$$

where α estimates the steady-state proportion of attached cross-bridges compared with the total number of cross-bridges. The ratio between stiffness in a force-generating state and stiffness in a rigor state (a state which reflects the maximum number of attached cross-bridges) can be used to estimate α (Brenner, 1988; Huxley, 1957). Knowing that rigor stiffness would be similar between different experimental conditions at the same SLs (SL of 2.4 µm at steady

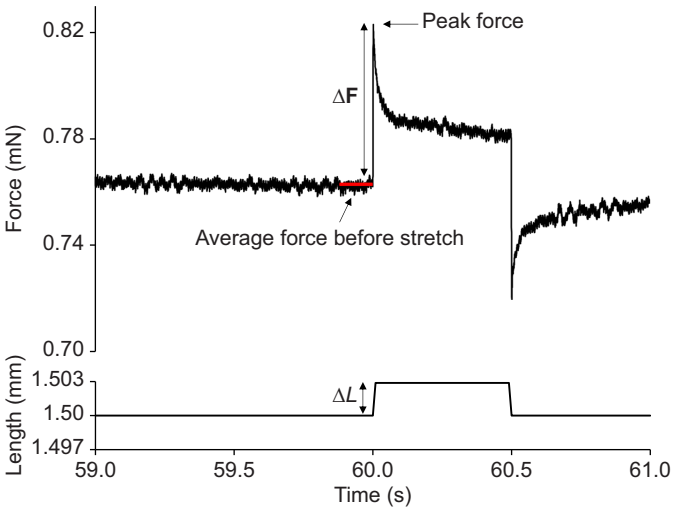

Fig. 2. Force as a function of time for the quick stretch–release cycle performed to determine fibre stiffness. Stiffness was determined as ΔF (peak force–average force before stretch) divided by ΔL (change in fibre length).

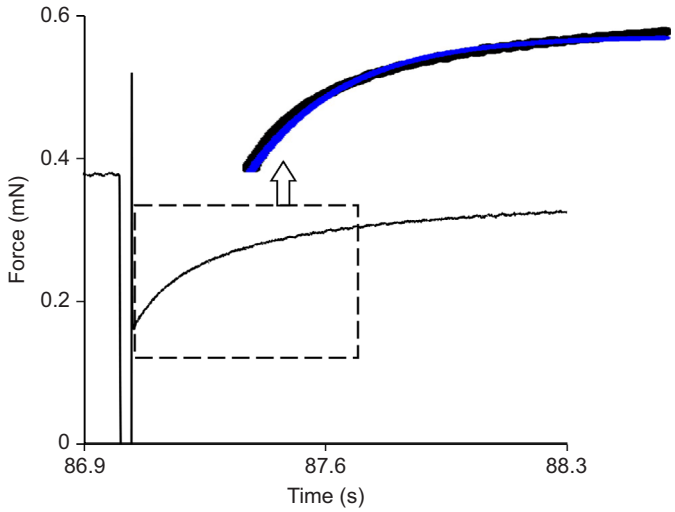

Fig. 3. Force as a function of time for the quick release–re-stretch protocol performed to determine k_{TR}. A mono-exponential regression curve (blue) fitted on the force redevelopment trace (black) to determine k_{TR} is shown.

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state following active shortening and its corresponding purely isometric contraction, and SL of 3.0 µm at steady-state following active stretching and its corresponding purely isometric contraction) and as adding additional tests to the procedure would increase the possibility of damaging the fibres, rigor stiffness was not determined in these experiments, but instead given an arbitrary value. We selected 100 mN mm^{-1} as the arbitrary rigor stiffness value, as using 100 mN mm^{-1} resulted in α values of~0.15–0.2 for the fibres in this experiment, which is consistent with findings that suggest 15–20% of available cross-bridges are attached to actin in a force-generating state during maximal isometric contractions (Finer et al., 1994). As such, α was calculated using:

$$\alpha = \frac{\text{Stiffness in a given condition}}{\text{Rigor stiffness}}. \quad (3)$$

The rate of force redevelopment following the quick release–re-stretch protocol reflects the distribution of cross-bridges between the attached force-generating and detached non-force-generating states, and thus, is associated with f and g according to Eqn 4 (Brenner, 1988):

$$k_{TR} = f + g, \quad (4)$$

where k_{TR} was experimentally determined as mentioned above.

Manipulation of Eqns 2 and 4 allowed f and g to be calculated using k_{TR} and α:

$$f = \alpha \times k_{TR}, \quad (5)$$

$$g = k_{TR} - f. \quad (6)$$

Note that f and g determined using these equations are only valid to determine differences in cross-bridge kinetics between states. Furthermore, as mentioned above, f and g are only the apparent rate constants of cross-bridge kinetics and are not the true rate constants of actin and myosin interactions (Brenner, 1988).

Force per cross-bridge

Stiffness is well-established as an indirect measure of the proportion of attached cross-bridges in single muscle fibres (Ford et al., 1981); therefore, an indirect estimation of force produced per cross-bridge was obtained by calculating the ratio of isometric force to stiffness (Kawai and Zhao, 1993).

Statistics

SPSS (v. 26, 2019) was used to determine significance between groups. Outcome measures were compared between the active shortening and stretching states and their corresponding reference states utilizing a Wilcoxon matched-pair test (P<0.05). Results are shown as means±s.e.m.

RESULTS

Active shortening experiments

Force and stiffness

Force and stiffness obtained at steady-state following active shortening were significantly depressed (15.4±1.5% and 11.2±2.2%, respectively) compared with the isometric reference contraction (P=0.001 and 0.008, respectively) (Fig. 4).

k_{TR}, f and g

k_{TR}, f and g were significantly depressed (4.6±1.3%, 11.7±3.3% and 4.2±1.3%, respectively) after active shortening compared with the isometric reference contraction (P=0.005, 0.002 and 0.002, respectively) (Fig. 5). Furthermore, the decrease in g was significantly smaller than the decrease in f (P=0.006).

Force per cross-bridge

The force to stiffness ratio was significantly depressed (3.9±2.3%) after active shortening compared with the isometric reference contraction (P=0.015) (Fig. 6).

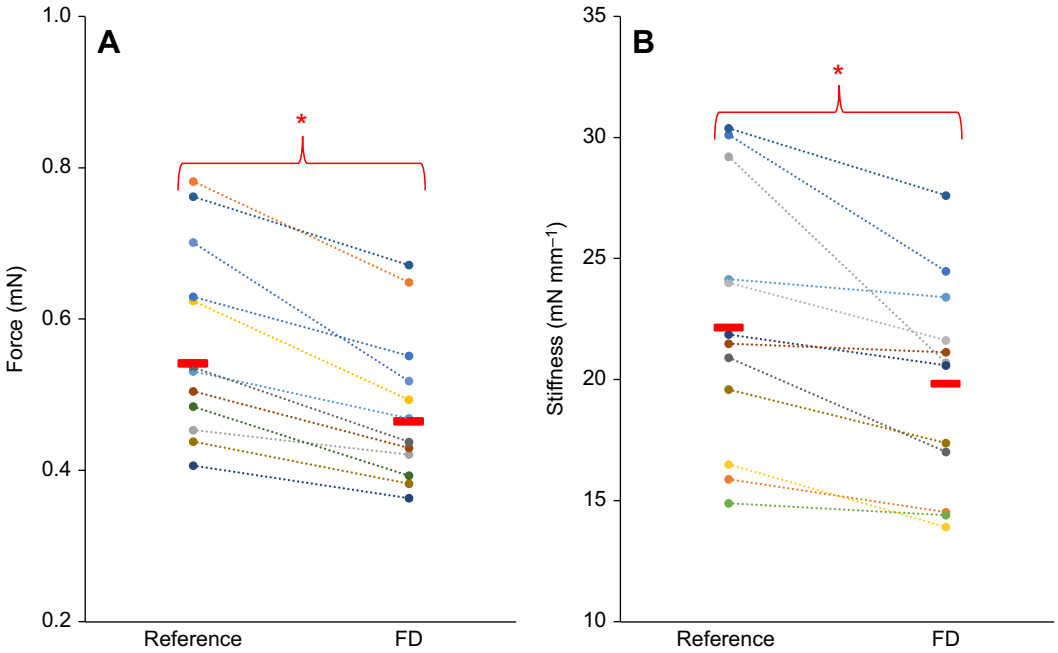

Fig. 4. Force and stiffness at steady-state following purely isometric and active shortening contractions. (A) Force and (B) stiffness for all 12 fibres tested. The dotted lines match the reference and active shortening (FD) values for a given fibre. The red dashes indicate the mean force and stiffness for the reference and FD conditions. *P<0.05, Wilcoxon matched-pair test.

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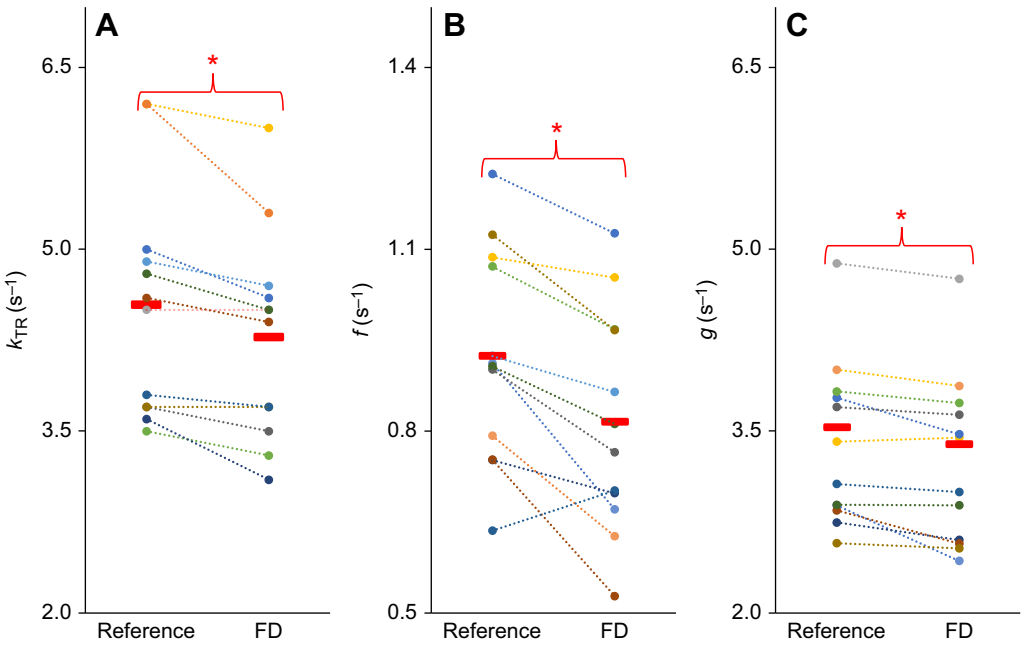

Fig. 5. k_{TR}, f and g at steady-state following purely isometric and active shortening contractions. (A) k_{TR}, (B) f and (C) g were determined at steady-state after purely isometric (reference) and FD contractions for all 12 fibres tested. The dotted lines match the reference and FD values for a given fibre. The red dashes indicate the mean values for the reference and FD conditions. *P<0.05, Wilcoxon matched-pair test.

Active stretch experiments

Force and stiffness

Force obtained at steady-state following active stretching was significantly enhanced (10.2±0.5%) compared with the isometric reference contraction (P=0.001) (Fig. 7A). Stiffness at steady-state after active stretching was not significantly different from the stiffness of the isometric reference contraction (P=0.714) (Fig. 7B).

k_{TR}, f and g

k_{TR}, f and g were not significantly different after active stretching compared with the isometric reference contraction (P=0.621, 0.177 and 0.158, respectively) (Fig. 8).

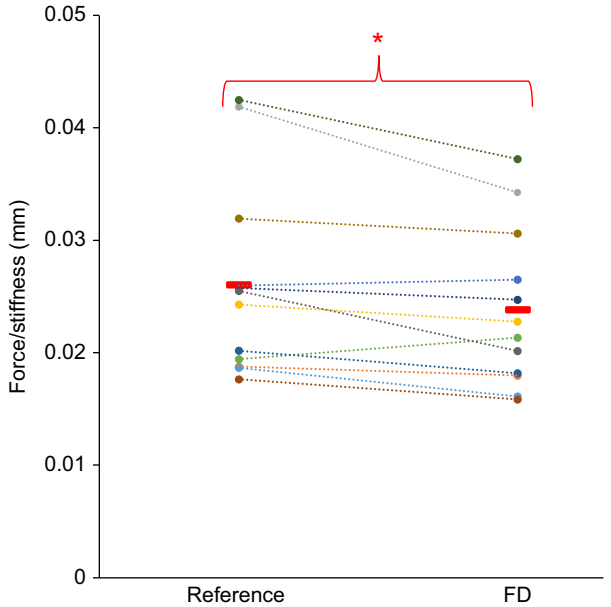

Fig. 6. Force to stiffness ratio at steady-state following purely isometric and active shortening contractions. The dotted lines match the reference and FD values for a given fibre. The red dashes indicate the mean force/stiffness for the reference and FD conditions. *P<0.05, Wilcoxon matched-pair test.

Force per cross-bridge

The force to stiffness ratio was significantly enhanced (11.1±1.3%) after active stretching compared with the isometric reference contraction (P=0.002) (Fig. 9).

DISCUSSION

Although changes in cross-bridge kinetics have been suggested to explain FD and FE, experiments elucidating cross-bridge kinetics in actively shortened and stretched muscle preparations and their corresponding purely isometric contractions have yet to be performed. The aim of this study was to investigate muscle cross-bridge cycling kinetics at steady-state following active shortening and stretching compared with the purely isometric reference contractions at the same final length and level of activation. Our results show, for the first time, that FD, but not FE, is accompanied by significant changes in cross-bridge kinetics. Specifically, a decrease in the rate of force redevelopment (k_{TR}), stiffness, rate of cross-bridge attachment (f), rate of cross-bridge detachment (g) and the force produced per cross-bridge was observed in the FD state, whereas FE was achieved without changes in k_{TR}, stiffness, f or g, but with an increase in the force produced per cross-bridge.

Force depression

All fibres showed significant FD at steady-state following active shortening compared with the purely isometric contractions at the same final length (Fig. 4A). This result agrees with previous observations of FD in skeletal muscle (for review, see Herzog, 2017). Stiffness decreased following active shortening compared with the purely isometric contractions (Fig. 4B), supporting previous findings in single fibre (Joumaa et al., 2021; Sugi and Tsuchiya, 1988), whole muscle preparations (Morgan et al., 2000) and *in vivo* human muscle (Lee and Herzog, 2003). Assuming the stiffness of actin, myosin and titin remains constant between the active shortening and reference states, it can be assumed that changes in stiffness are primarily caused by a change in the proportion of attached cross-bridges between conditions (Joumaa et al., 2021; Lee and Herzog, 2003; Sugi and Tsuchiya, 1988).

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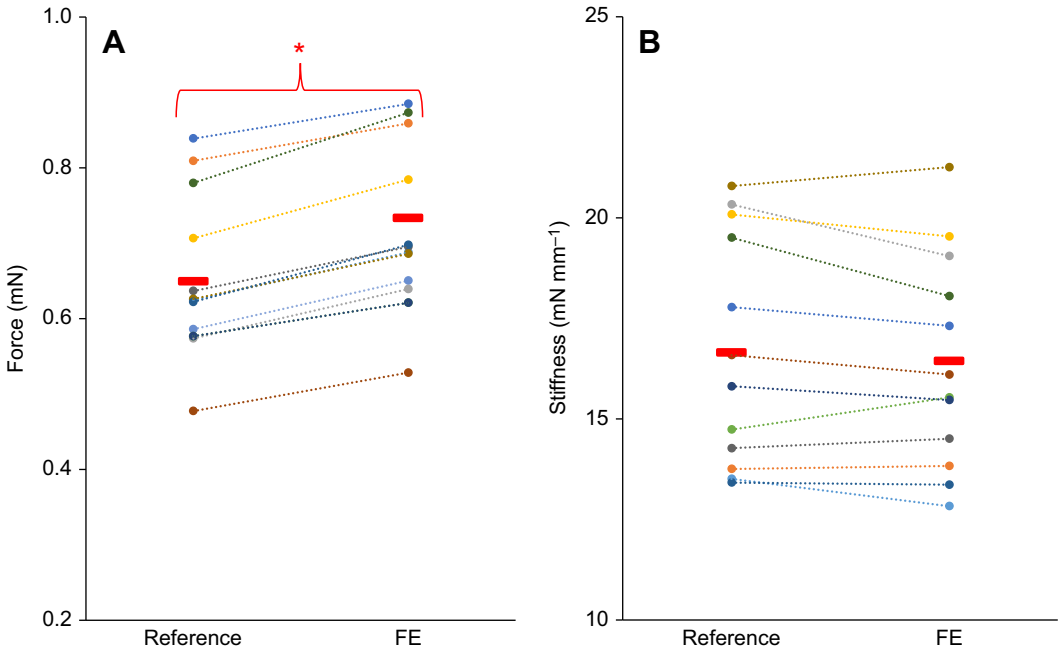

Fig. 7. Force and stiffness at steady-state following purely isometric and active stretch contractions. Force (A) and stiffness (B) at steady-state following purely isometric (reference) and active stretch (FE) contractions for all 12 fibres tested. The dotted lines match the reference and FE values for a given fibre. The red dashes indicate the mean force and stiffness for the reference and FE conditions. *P<0.05, Wilcoxon matched-pair test.

Therefore, it appears that actively shortened muscle fibres have a decreased steady-state proportion of attached cross-bridges compared with their purely isometric counterparts.

The rate of force redevelopment at steady-state following active shortening was significantly decreased compared with the purely isometric contraction at the same final length (Fig. 5A). This result is in accordance with our hypothesis and indicates that a change in cross-bridge cycling kinetics is occurring at steady-state isometric contraction in the actively shortened fibres. The rate constants, f and g, determined at steady-state following active shortening, were significantly decreased compared with the purely isometric contractions at the same final length (Fig. 5B,C). Interestingly, the decrease in the rate of cross-bridge detachment was significantly less than the decrease in the rate of cross-bridge attachment

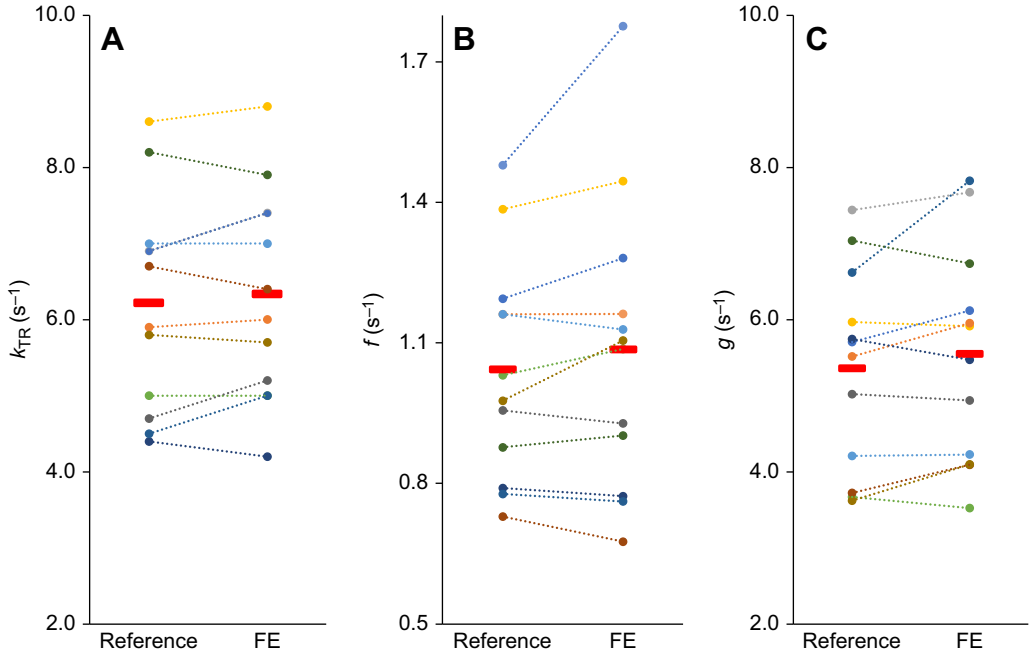

Fig. 8. k_{TR}, f and g at steady-state following purely isometric and active stretch contractions. (A) k_{TR}, (B) f and (C) g were determined at steady-state after purely isometric (reference) and FE contractions for all 12 fibres tested. The dotted lines match the reference and FE values for a given fibre. The red dashes indicate the mean values for the reference and FE conditions. There was no significant difference in k_{TR}, f or g between conditions. P>0.05, Wilcoxon matched-pair test.

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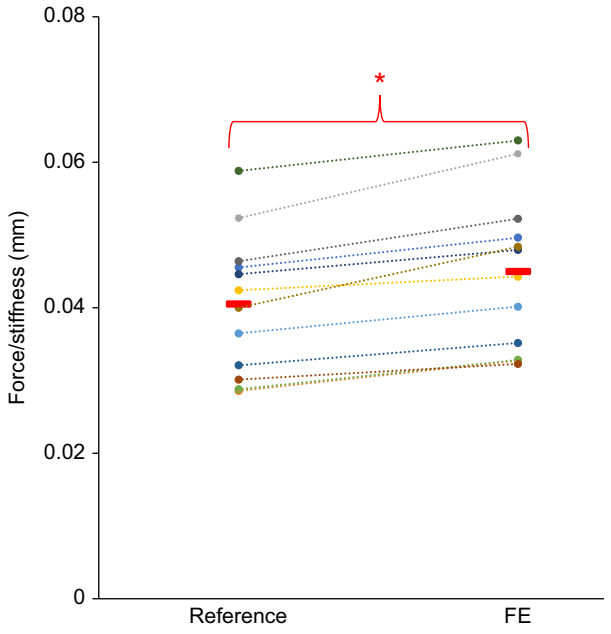

Fig. 9. Force to stiffness ratio at steady-state following purely isometric and active stretch contractions. The dotted lines match the reference and FE values for a given fibre. The red dashes indicate the mean force/stiffness for the reference and FE conditions. *P<0.05, Wilcoxon matched-pair test.

(4.2±1.3% and 11.7±3.3%, respectively); therefore, it is unlikely that the decrease in cross-bridge detachment could compensate for the large decrease in cross-bridge attachment. The combined change in f and g could be a factor leading to the decrease in the proportion of attached cross-bridges and FD observed in actively shortened muscle fibres.

We observed a decrease in the ratio of force to stiffness at steady-state following active shortening (Fig. 6), suggesting that the force produced per cross-bridge decreases at steady-state following active shortening compared with the purely isometric contraction at the same final length. A potential explanation for this decrease in force produced per cross-bridge could be that, in addition to the changes in cross-bridge kinetics suggested by our k_{TR}, f and g results, the cross-bridges are undergoing a change in their conformation leading to a decrease in the amount of strain in myosin subfragment 2 (S2) segments that are responsible for tethering myosin to actin and producing active force (for review, see AL-Khayat, 2013). Therefore, FD observed at steady-state in actively shortened fibres compared with their corresponding purely isometric contractions is likely the combined result of a reduction in the force produced per cross-bridge, and the proportion of attached cross-bridges.

The mechanism responsible for the decrease in the proportion of attached cross-bridges, and the force produced per cross-bridge following active shortening remains unclear. It has been suggested that active shortening may be associated with a stress-induced inhibition of cross-bridge attachment in the new actin-myosin overlap zone formed after active shortening (Maréchal and Plaghki, 1979). According to this hypothesis, actin filaments in exclusively actin (I-band) regions are strained upon force production, potentially producing conformational changes in the myosin attachment sites, and inhibiting normal attachment of cross-bridges. We suggest that the stress during active shortening could result in a change in the conformation of cross-bridges and thus reduce the force produced per cross-bridge as well as inhibit cross-bridge formation. Further experiments to indicate if conformational changes occur in actin alone, myosin alone, or in both, and how these changes may affect the cross-bridge kinetics, would provide useful information as to the mechanism responsible for the changed cross-bridge kinetics and reduced force at steady-state following active shortening.

Force enhancement

All fibres showed significant FE at steady-state following active stretching, compared with the purely isometric contractions at the same final length (Fig. 7A). This result aligns with previous observations of FE in skeletal muscle preparations ranging from single myofibrils to human muscles *in vivo* (for review, see Herzog, 2017). Stiffness after active stretch was not significantly different from the stiffness in the purely isometric contractions at the same final length (Fig. 7B), supporting previous findings (Joumaa and Herzog, 2013; Joumaa et al., 2021; Sugi and Tsuchiya, 1988). The absence of a change in stiffness after active stretching suggests that the increase in steady-state force following active stretch is not achieved by an increase in the proportion of attached cross-bridges compared with the purely isometric contractions. Similarly, no change in k_{TR}, f or g between the active stretch and purely isometric contractions was observed (Fig. 8). This suggests that cross-bridge cycling kinetics did not change between conditions and that the origin of FE is not associated with the cross-bridge cycling kinetics.

The increase in force to stiffness ratio observed in actively stretched muscle fibres suggests that an increase in the force produced per cross-bridge may be a potential mechanism leading to FE (Fig. 9). However, it seems that for a cross-bridge to produce more force, a conformational change in one, or various cross-bridge components would need to occur. This conformational change would likely impact the cross-bridge kinetics in some way, and as no significant change in the rate of cross-bridge attachment or detachment was observed when comparing actively stretched contractions to the corresponding purely isometric reference contractions, it is unlikely that FE occurs due to a change in the force produced per cross-bridge. Furthermore, the force produced per cross-bridge was not directly measured in these experiments but was calculated as the total force produced by the fibre divided by fibre stiffness. Total force produced includes active force and passive force. Ideally, the force produced per cross-bridge should be calculated from active force alone because this force originates from the cross-bridges, as opposed to passive force, which results from structures outside the cross-bridges. We believe that the observed increase in the force produced per cross-bridge obtained in this study results from an increase in passive force following active stretching and not from active force and the cross-bridges themselves.

Indeed, a more likely mechanism leading to FE is the engagement of a passive element in actively stretched fibres that provides a passive force in addition to the active force produced from the attached cross-bridges (Edman et al., 1978; Herzog and Leonard, 2002; Hessel et al., 2017; Noble, 1992; Pinniger et al., 2006). The titin filament present in a sarcomere is a likely candidate to provide this passive force (Horowits et al., 1986). Titin has been characterized as being a molecular spring that provides a force resisting the stretch of muscle fibres (Linke and Granzier, 1998). It has been shown that when a muscle is activated, calcium binds to titin (Tatsumi et al., 2001) and increases its stiffness when actively lengthened (DuVall et al., 2013; Joumaa et al., 2008b; Labeit et al., 2003). Furthermore, Leonard and Herzog (2010) showed that when myofibrils were actively stretched to sarcomere lengths beyond myofilament overlap, where cross-bridge formation and active force production was impossible, force remained 2- to 3-times greater

than the passive forces for myofibrils stretched passively to the same lengths. In these experiments, the calcium-based increase in titin stiffness was not sufficient to explain the tremendous increase in force observed following active stretching to sarcomere lengths beyond myofilament overlap. They proposed a theory whereby titin binds to actin in actively stretched muscles, thus effectively shortening the structural protein and increasing its stiffness when actively stretched compared with titin's stiffness when passively stretched (Dutta et al., 2018; Kellermayer and Granzier, 1996; Leonard and Herzog, 2010; Linke et al., 1997). Although the results of this study do not provide direct evidence for titin's involvement in FE, the absence of changes in cross-bridge kinetics following active stretching suggests that a passive element, outside the cross-bridges, contributes to FE. The likely candidate for this role is titin. The mechanism by which titin, or another passive element, contributes to FE, needs further investigation.

Limitations

A limitation of this study was the use of a two-state cross-bridge model, and many assumptions, in the characterization of k_{TR}, f and g. We assumed that cross-bridges exist in two states (attached and force-producing cross-bridges, detached and non-force-producing cross-bridges) in a muscle fibre. This assumption is useful for cross-bridge modelling; however, it fails to account for other characterized states of cross-bridges, such as weakly attached, non-force-producing cross-bridges (Eisenberg and Hill, 1985). In other studies, multiple cross-bridge state models have been used to fit actively shortened and actively stretched force redevelopment traces with a double-exponential model to provide two rates of force re-development. The fast, initial re-development is associated with the rapid transition of attached cross-bridges from weakly bound to strongly bound states (Huxley and Simmons, 1971) and the slower, later re-development is associated with the formation of new cross-bridges (Huxley and Simmons, 1971; Koppes et al., 2013, 2015). The results of double-exponential regression model fitting of force redevelopment in our research were consistent with the results of the mono-exponential regression model fitting. Despite this, fitting of the fast, initial re-development was not always accurate, and tended to decrease the correlation between the fitting curve and the experimental data. Therefore, results from the mono-exponential model were presented for our study.

Another limitation resides in the calculation of the fraction of attached cross-bridges (α). As we used a two-state cross-bridge model (attached force-generating and detached non-force-generating cross-bridges, without considering the weakly bound cross-bridges) to calculate the rates of force attachment and detachment, we estimated the fraction of attached cross-bridges based on experiments by Finer et al. (1994), as opposed to using the ratio between our active stiffness values, and rigor stiffness values obtained from either an additional set of fibres or from the literature. Finer et al. (1994) measured the force produced per cross-bridge in single myosin and actin filament experiments and then estimated the proportion of attached force-generating cross-bridges based on the force produced per cross-bridge and the total force produced by fibres/muscles. Therefore, Finer et al.'s measurements provide a better estimation of the ratio of attached force generating cross-bridges for a two-state cross-bridge model than stiffness measurements which include weakly bound cross-bridges. Furthermore, we assumed that the rate constants f and g were the same for all cross-bridges. Some regions of a muscle fibre, such as the newly formed overlap regions of myosin and actin filaments that occur with active shortening, may have different rates of cross-bridge attachment and detachment compared with other regions of the muscle fibre (Maréchal and Plaghki, 1979) and cross-bridge cycling kinetics has also been shown to be potentially force-dependent (Hooijman et al., 2011).

The release–re-stretch protocol may have impacted FD and FE properties. FD and FE have been shown to be abolished when force was allowed to drop to 'zero' at steady-state following active shortening and stretching, respectively, in cat soleus muscle (Herzog and Leonard, 1997, 2002). In our experiments, FD and FE were not abolished after the release–re-stretch protocol (result not shown), which initially reduces force to 'zero'. This may be a potential limitation of the shortening–re-stretch cycle and warrants further research to understand the effect of active shortening, stretching, and shortening–re-stretching cycles on FD and FE.

Conclusion

In summary, we have shown for the first time that a change in cross-bridge cycling kinetics and a decrease in the force produced per cross-bridge accompany FD, which could potentially be a mechanism leading to the characterized decrease in force following active muscle shortening conditions compared with the corresponding purely isometric contractions. However, no association was found between FE and changes in cross-bridge kinetics, which provides support for the hypothesis that FE does not originate from altered cross-bridge kinetics but likely from passive structures.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.R.A., V.J., W.H.; Methodology: S.R.A., V.J., W.H.; Validation: V.J.; Formal analysis: S.R.A., V.J.; Investigation: V.J., S.R.A.; Resources: V.J., W.H.; Data curation: S.R.A., V.J.; Writing - original draft: S.R.A.; Writing - review & editing: S.R.A., V.J., W.H.; Visualization: S.R.A.; Supervision: V.J., W.H.; Project administration: W.H.; Funding acquisition: W.H.

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